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The Role of a Trypsin-Sensitive Factor in Multiplicity Reactivation with Heat-Disrupted Phage

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(*Received 25 July 1960*)

Heat-disrupted T2 or T4 phage can infect *E. coli* spheroplasts. Multiplicity reactivation can take place in spheroplasts infected with heat-disrupted T2 or T4 phage which have been irradiated with ultraviolet light. Such reactivation failed to occur with trypsin digested preparations. Similar results were obtained in spheroplasts cross-infected with disrupted T2 and T4 phage; thus the reactivation of T2 phage which has been irradiated with ultraviolet light, heated and then digested with trypsin, can be observed in spheroplasts simultaneously infected with heated T4 phage. The reactivating ability of this heated T4 phage was greatly inhibited by digestion with trypsin but not with deoxyribonuclease or ultraviolet light irradiation.

From these results it can be inferred that the trypsin-sensitive factor, present in phage DNA preparations, is required for multiplicity reactivation and that multiplicity reactivation of disrupted T2 phage can also take place with the trypsin-sensitive factor from T4 phage DNA preparations.

1. Introduction

It has been shown that disrupted T2 phage can infect *E. coli* spheroplasts that are resistant to infection by intact phage (Spizizen, 1957; Fraser, Mahler, Shug & Thomas, 1957). Using this system, we can hope that the mechanism of phage multiplication or the chemical analysis of infecting phage DNA will be more amenable to study than the normal infection system. In this spheroplast system, the presence of protein in infecting phage DNA preparations seemed to be essential to the production of phage particles since the activity of phage DNA was lowered by trypsin digestion (Spizizen, 1957; Sekiguchi, 1958; Mahler & Fraser, 1959; Kiho & Watanabe, 1960).

The presence of protein, which is different from that of the phage coat, has been demonstrated in osmotic shockates of T2 by chemical (Hershey, 1957) and immunological methods (Levine, Barlow & Van Vunakis, 1958; Murakami, Van Vunakis & Levine, 1959).

We have attempted to gain some insight into the role of the trypsin-sensitive factor in multiplication of phage in spheroplasts infected with disrupted phage. Our previous studies of the initial reaction† of heat-disrupted phage (Kiho, Haruna & Watanabe, 1959), and of multiplicity reactivation in this system, have led us to consider the possibility that the trypsin-sensitive factor may be required for the construction of phage chromosome (Kiho & Watanabe, 1960). In this communication,

† See Watanabe & Kiho (1957), for studies on the initial reaction in T2-infected *E. coli*.

the requirement of the trypsin-sensitive factor in MR† will be further substantiated by experiments using spheroplasts cross-infected with disrupted T2 and T4 phage and attempts will be made to define the necessary condition for MR to occur in this system.

2. Materials and Methods

A wild type *E. coli* strain B(H) and its derivative B/4 resistant to T4 phage were used. Spheroplasts were prepared from *E. coli* B(H) grown in tris-glucose medium‡ containing 0·05% Difco Casamino acids according to the method of Fraser *et al.* (1957) and were suspended in tris medium containing 0·125 M-sucrose.

Bacteriophage T2r^+ and T4r, suspended in tris medium were used. Heat-disruption of phage was performed at 70 to 71°C for 15 min.

Trypsin digestion of this heated phage was carried out by incubating the sample with 10 μg/ml. trypsin at 37°C for 60 min.

Deoxyribonuclease digestion was performed by incubating heated T4 (1×10^{12} phage equivalents/ml. suspended in 4 times H_2O-diluted tris medium containing 0·002 M-$MgSO_4$) with various concentrations of DNase at 37°C for 60 min.

Ultraviolet light irradiation was carried out in dim light with a 15 watt "germicidal lamp" at a distance of 80 cm. After u.v. irradiation the phage was heated and then, when necessary, trypsin or DNase digestion was carried out as described above.

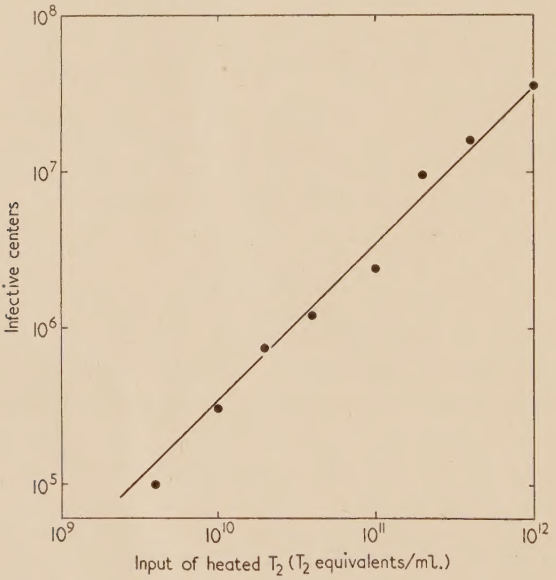

FIG. 1. Infectivity of heat-disrupted phage. One volume of heated T2 phage was added to nine volumes of spheroplasts (3×10^8/ml.), held for 30 min at 37°C for adsorption, diluted into peptone-glucose medium and assayed for infective centers.

† The following abbreviations are used in this report:

MR: multiplicity reactivation
H : heated
T: trypsin digested
U: u.v.-irradiated
D: DNase digested

‡ Tris medium contains per liter 5·4 g NaCl, 3 g KCl, 1·1 g NH_4Cl, 0·1 mM-$CaCl_2$, 1 mM-$MgCl_2$, 0·1 M-2-amino-2-hydroxymethylpropane-1:3 diol adjusted to pH 7·2 at 37°C, 20 mg phosphorus (as KH_2PO_4) and 6 mg sulfur (as Na_2SO_4).

Tris-glucose medium is tris medium plus 0·2% glucose.

3. Results and Discussion

(a) *The infectivity of heat-disrupted phage*

The infectivity of heat-disrupted phage is shown in Fig. 1. It can be seen that the logarithm of the number of infective centers after adsorption onto spheroplasts increases linearly with the logarithm of the concentration of the input of disrupted phage. As reported previously (Kiho & Watanabe, 1960), trypsin digestion has the effect of increasing the eclipse period of T2 multiplication in this system without

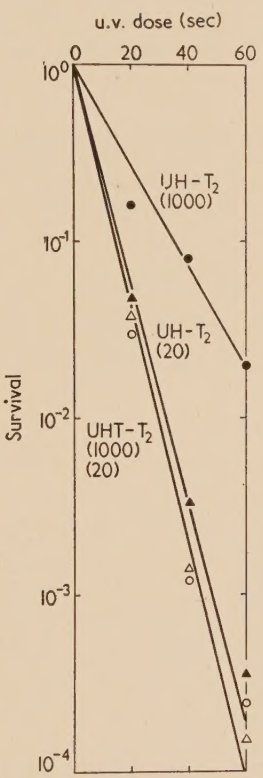

FIG. 2. Multiplicity reactivation of heat-disrupted T2 phage. The survival of infective centers after adsorption onto spheroplasts has been plotted against u.v. dose. Spheroplasts (1×10^8/ml.) were infected with various disrupted T2 preparations at the following input ratio:

preparation	input ratio (T2 equivalents per spheroplast)
● UH-T2	1000
▲ UH-T2	20
○ UHT-T2	1000
△ UHT-T2	20

After adsorption onto spheroplasts, infective centers were assayed.

affecting the number of infective centers after adsorption to spheroplasts. As the number of infective centers after adsorption is related to the amount of input disrupted phage under the conditions used, this means that the trypsin-sensitive factor may be required for the multiplication process itself after DNA injection into spheroplasts. (In the case of heated T2 prepared at a higher temperature, trypsin digestion

was also observed to have the effect of decreasing the number of infective centers.) The presence of 10 μg/ml. trypsin at or after the time of adsorption did not have any effect.

(b) *Multiplicity reactivation of heat-disrupted phage*

As reported previously (Kiho & Watanabe, 1960), MR could take place in spheroplasts infected with u.v. irradiated, heated T2 (UH-T2) but not with its trypsin digest (UHT-T2). This is shown in a simplified form in Fig. 2. Furthermore, it can be seen that in the experiments with low multiplicity, the slope of UH-T2 approaches that of UHT-T2. From these results, it may be inferred that the trypsin-sensitive factor is required for MR. The same observations were made with heat-disrupted T4 phage.

(c) *Reactivation of UHT-T2 by heat-disrupted T4 phage*

To show the requirement for the trypsin-sensitive factor in MR more clearly we then studied the reactivation of UHT-T2 by various disrupted phage preparations.

TABLE 1

Reactivation of UHT-T2 by disrupted T4

Addition				T2		T4
Disrupted T2		Disrupted T4		Titer	R‡	Titer
UHT	200†	H	1600†	$6{\cdot}2\times10^{3}$	25	—
,,	,,	HT	,,	$1{\cdot}2\times10^{3}$	5	—
,,	,,	UH	,,	$4{\cdot}0\times10^{3}$	16	—
,,	,,	UHT	,,	$2{\cdot}8\times10^{2}$	1	—
—		H	1600	—		$1{\cdot}4\times10^{7}$
—		HT	,,	—		$3{\cdot}0\times10^{6}$
—		UH	,,	—		$2{\cdot}1\times10^{5}$
—		UHT	,,	—		$4{\cdot}0\times10^{2}$
UHT	200	—		$2{\cdot}5\times10^{2}$		—
UH	,,	—		$1{\cdot}1\times10^{3}$		—

Spheroplasts (1×10^{8}/ml.) were infected with UHT-T2 (u.v. dose: 90 sec at 80 cm) and disrupted T4 phage (u.v. dose: 180 sec at 80 cm). After adsorption onto spheroplasts infective centers were determined.

† input ratio = phage equivalents/spheroplast

‡ R = degree of reactivation of UHT-T2 (reactivated T2/titer of UHT-T2)

In order to distinguish the reactivated T2 phage from the latter (reactivator), we have used disrupted T4 phage as the reactivator and B/4 as plating indicator to detect T2 phage. (The content of T4*h* in our T4 phage preparation is of the order of 10^{-6} and does not influence the results.)

As shown in Table 1, the reactivation of UHT-T2 could occur in spheroplasts simultaneously infected with H-T4. With u.v. irradiated T4 preparations (UH-T4), this reactivation of UHT-T2 was not much affected, but it was strongly inhibited by

trypsin digestion. Furthermore, when we compare the reactivation by HT-T4 with that by UH-T4, it can be seen that the degree of T2 reactivation does not always run parallel with the infectivity of disrupted T4 preparations. These results suggest the following:

(i) The degree of reactivation cannot always be judged from the infectivity of disrupted T4. In particular, the trypsin treated preparation seems to be quite different from the untreated control with respect to reactivating activity. (Another qualitative difference between H-T2 and HT-T2 could be derived from the study of intracellular phage growth, as reported previously (Kiho & Watanabe, 1960).)

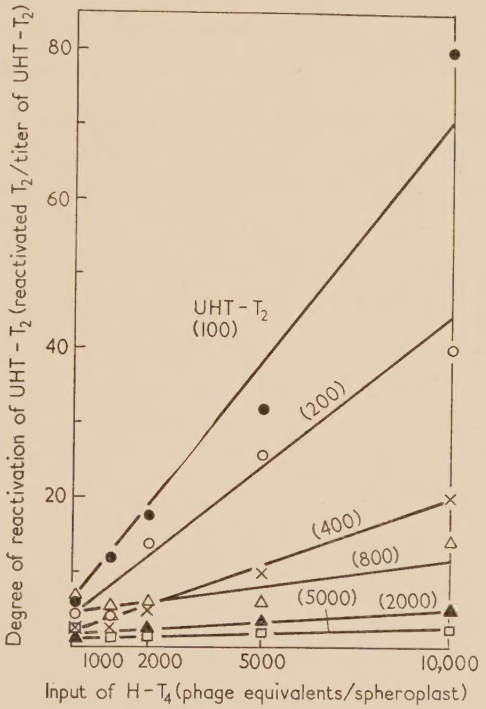

FIG. 3. Reactivation of UHT-T2 by H-T4. The degree of UHT-T2 reactivation (u.v. dose: 90 sec at 80 cm) plotted against the input H-T4 at various concentrations of UHT-T2. Spheroplasts (1×10^8/ml.) were mixedly infected with UHT-T2 and H-T4. After adsorption onto spheroplasts, the reactivated T2 phage was assayed on B/4.

Input of UHT-T2 (phage equivalents per spheroplast)	
●	100
○	200
×	400
△	800
▲	2000
□	5000

(ii) The reactivator is not the intact phage, present in disrupted phage preparations. (The titer of the intact phage was not affected by trypsin digestion and in fact, this titer was so low that the intact phage have no influence upon the results.)

In addition to these results, when we compare the titer of T2 reactivated by H-T4 or UH-T4 with that of UH-T2 alone, it can be seen that the former has a higher

value than the latter. From this fact it does not seem that the reactivation of UHT-T2 is merely the rescue of trypsin damage. In further experiments, evidence has been obtained that u.v. damage itself may be rescued. Details of these experiments will be published separately.

As described above, the degree of reactivation of UHT-T2 cannot always be judged by the infectivity of the reactivator when using different kinds of disrupted T4 preparations. However, with the same kind of preparation the reactivation depends upon the concentration of the reactivator, as shown in Fig. 3, in which the degree of

FIG. 4. Reactivation of UHT-T2 by H-T4. The degree of reactivation of UHT-T2 plotted against the relative content of H-T4 from the data in Fig. 3. The same symbols were used as in Fig. 3.

UHT-T2 reactivation is plotted against the input of H-T4 at various concentrations of UHT-T2. It can be seen that at a relatively low concentration of UHT-T2 the reactivation depends upon the concentration of input H-T4, but at higher concentrations of UHT-T2 the reactivation cannot be demonstrated clearly. In Fig. 4 the degree of reactivation of UHT-T2 has been re-plotted against the relative content of H-T4 (amount of H-T4/amount of UHT-T2). From this graph it can be seen that the reactivation of UHT-T2 depends on the relative amount of reactivator. In these experiments, although we used relatively high concentrations of disrupted phage, no interference between these phages could be observed.

(d) *Reactivation of UHT-T2 by DNase digested preparations*

To verify the inference that the trypsin-sensitive factor is required for MR, it would be necessary to show that the reactivation of UHT-T2 occurs in spheroplasts

mixedly infected with UHT-T2 and a preparation of the isolated factor. Up to the present, we have not been able to succeed in doing this. However, it can be shown that reactivation of UHT-T2 is also produced by DNase treated preparations.

Figure 5 shows the results of these experiments, in which the following data are included:

(i) Survival of UHD-T4 plotted against the concentration of DNase used for pretreatment of UH-T4 (curve A).

(ii) Degree of reactivation of UHT-T2 by these UHD-T4 (curve B).

(iii) In order to see whether the above reactivation is due to the undamaged fraction of UHD-T4 preparations a control experiment was performed in which reactivation by UH-T4, diluted to give the same infectivity as UHD-T4, was studied (curve C). It can be seen that the slope of the curve in UHT-T2 reactivation by UHD-T4 is less steep than that of the curve in reactivation by diluted UH-T4. In particular, when

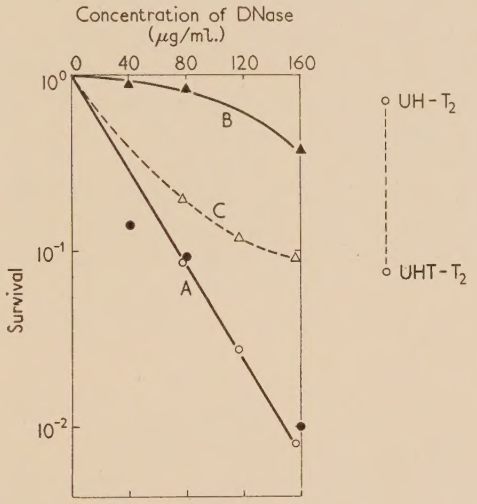

FIG. 5. Reactivation of UHT-T2 by UHD-T4. Spheroplasts (1×10^8/ml.) were mixedly infected with UHT-T2 (u.v. dose: 60 sec at 80 cm) and UHD-T4 (UH-T4 treated with various concentrations of DNase, shown in abscissa) or diluted UH-T4 (u.v. dose for T4: 180 sec at 80 cm). The input ratios of UHT-T2 and UHD-T4 were 750 and 2000 phage equivalents per spheroplast, respectively. After adsorption onto spheroplasts reactivated T2 phage were assayed on B/4. The infectivity of UHD-T4, UHT-T2 and UH-T2 was also shown.

curve A (●): survival of UHD-T4
curve B (▲): degree of reactivation of UHT-T2 by UHD-T4
curve C (△): control (see text)
(○): infectivity of diluted UH-T4 (see text)

we compare the reactivation by UHD-T4 with that by diluted UH-T4, both having an infectivity 1/100 that of the original UH-T4, it is apparent that reactivation by diluted UH-T4 can hardly occur while appreciable reactivation by UHD-T4 can be observed. Furthermore, the infectivity of UHD-T4 (160 μg/ml. DNase-digested preparation) was lower than that of UHT-T2. From these results it can be stated that reactivation of UHT-T2 may not be directly related to the T4 DNA, but to the trypsin-sensitive factor present in UHD-T4.

However, when higher concentrations of DNase were used for the preparation of UHD-T4, reactivation of UHT-T2 could not be observed. The possibility that this was due to the inhibition of phage growth by DNase carried over in the UHD-T4

preparation was excluded by the control experiments in which DNase was added after UH-T4 infection. At present it is not known why the reactivation by these UHD-T4 cannot be observed. Does the reactivation of UHT-T2 require some form of DNA-protein structure which may also be destroyed by exhaustive digestion with DNase, or does it require some portion of the T4 DNA? To answer these questions genetic analysis of reactivated T2 phage must be carrried out, including the studies on the mechanism of MR. As has been pointed out previously (Kiho & Watanabe, 1960), the recombination mechanism in relation to MR (Luria, 1947; Luria & Dulbecco, 1949; Baricelli, 1956) should take the possible role of the trypsin-sensitive factor into consideration. Studies along these lines are now in progress.

At present we do not know the relation between our trypsin-sensitive factor and peptides or proteins mentioned by other workers (Hershey, 1957; Levine *et al.*, 1958, Murakami *et al.*, 1959). As described above, our preparations of spheroplast-infecting agent were obtained by heating phage particles at relatively low temperatures without osmotic shocking. Judging from the small effect of trypsin digestion on the number of infective centers after adsorption onto spheroplasts, or on the phage yield after multiplication in this system (Kiho & Watanabe, 1960), it seems that our conditions of trypsin treatment of heated phage preparations is milder than those employed by other workers (Spizizen, 1957; Sekiguchi, 1958; Mahler & Fraser, 1959). Nevertheless, multiplicity reactivation did not occur with the trypsin digested preparations. From these facts it seems that the effect of trypsin digestion on multiplicity reactivation may be more localized than that on phage infectivity.

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REFERENCES

Baricelli, N. A. (1956). *Acta Biotheor.* **11**, 107.
Fraser, D., Mahler, H. R., Shug, A. L. & Thomas, C. A., Jr. (1957). *Proc. Nat. Acad. Sci., Wash.* **43**, 939.
Hershey, A. D. (1957). *Virology*, **4**, 237.
Kiho, Y., Haruna, I. & Watanabe, I. (1959). *Progr. in Virus Res.* (Japan), **2**, 55 (in Japanese).
Kiho, Y. & Watanabe, I. (1960). *J. Mol. Biol.* **2**, 78.
Levine, L., Barlow, J. L. & Van Vunakis, H. (1958). *Virology*, **6**, 702.
Luria, S. E. (1947). *Proc. Nat. Acad. Sci., Wash.* **9**, 253.
Luria, S. E. & Dulbecco, R. (1949). *Genetics*, **34**, 93.
Mahler, H. R. & Fraser, D. (1959). *Virology*, **8**, 425.
Murakami, W. T., Van Vunakis, H. & Levine, L. (1959). *Virology*, **9**, 624.
Sekiguchi, M. (1958). *Virology*, **6**, 77.
Spizizen, J. (1957). *Proc. Nat. Acad. Sci., Wash.* **43**, 694.
Watanabe, I. & Kiho, Y. (1957). *Int. Sym. Enz. Chem., Tokyo and Kyoto*, 418.

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Hydroxylamine as a Mutagenic and Inactivating Agent†

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(*Received 19 September 1960*)

Hydroxylamine (HA) inactivates T4 phages in two different ways. A non-mutagenic effect is strongest at an intermediate concentration of HA and a low concentration of salt, while high concentrations of both suppress this action. The second effect, observed only at high concentrations of HA and salt, is highly mutagenic.

The mutagenic action of HA produces mottled T4 *r* plaques under conditions of little inactivation, while for longer treatment more and more pure *r* plaques have been observed. This has been examined quantitatively. *r*II-type mutations are distributed over the whole *r*II region and 94% of these can be induced to revert by 2-aminopurine.

Certain nucleic acid bases treated with HA show a decrease in u.v. absorption while others gave no effect even when analysed by chromatography. The reacting bases are uracil, cytosine and 5-hydroxymethylcytosine; no reaction was found with purines, thymine, 5-methylcytosine and pseudouridine.

The preferential action of HA with cytosine, of the DNA bases, suggests the direction of the mutagenic effect.

1. Introduction

The direction of mutagenic base or base-pair changes in nucleic acids, as well as some functional properties of DNA, should be determinable if one analyses both the chemical and the genetic effect of certain reagents which attack different groups of the nucleic acid bases. So far, the effect of only a few chemicals has been examined in sufficient detail for their sites of attack on nucleic acids to be known. Alkyl sulfates react preferentially with the nucleophilic ring nitrogens of the bases (Reiner & Zamenhof, 1957; Brookes & Lawley, 1960) and can attach to the phosphates (Stacey, Cobb, Consens & Alexander, 1958), low pH removes purines (Tamm, Hodes & Chargaff, 1952), nitrous acid oxidatively deaminates the bases (e.g. Schuster & Schramm, 1958), hydrazine breaks and removes large portions of the pyrimidine rings (e.g. Takemura, 1959), and glyoxal derivatives react preferentially with guanine (Staehlin, 1958). All but the last two of these agents have been shown to be mutagenic.

This paper describes the lethal and mutagenic effect of hydroxylamine (HA) and shows that for free DNA bases and nucleosides it reacts predominantly with cytosine, while for RNA bases both uracil and cytosine are affected.

2. Materials and Methods

Phages: Standard T4 (Benzer) and *r*II mutants derived from it. λ reference type (Kaiser).

Bacteria: B = *E. coli* B (American); K = K12 (λ); C = C600 for plating of λ.

† This work has been supported by grants of the U.S. Public Health Service and the Damon Runyon Memorial Fund for Cancer Research. Paper no. 801 from the Department of Genetics, University of Wisconsin.

Broth: 1% Bacto-tryptone (Difco) plus 0·5% NaCl.
Dilution broth: 0·5% Bacto-tryptone plus 1 M-NaCl.
Buffer: 17·7g $Na_2HPO_4,12H_2O$; 3 g KH_2PO_4; 10^{-3} M-$MgSO_4$; 1 liter H_2O.
Pseudouridine: was a gift of Dr. Maxine Singer.
Hydroxylamine medium: NH_2OH,HCl was dissolved in 2 × conc. buffer and the pH adjusted to 7·5 with conc. NaOH, H_2O or NaCl were then added until the desired concentration of buffer, NH_2OH and NaCl was obtained and the pH adjusted once more. NaOH was added only a few minutes before the start of the experiment.
Method of phage inactivation: Phages were added to cold hydroxylamine medium and a few seconds allowed for equilibration. A portion was then diluted in dilution broth and plated with 2×10^8 freshly grown bacteria B; this gave the zero time control. The remainder was placed in a 37°C waterbath (= zero time) and at different times samples were diluted and plated on B.
The genetic methods have been described earlier: for the mapping with deletion mutants see Benzer & Freese (1958) and for reversion induction by base analogues see Freese (1959).
The u.v. absorption of the reacting bases was measured in a Zeiss spectrophotometer at the wavelength of maximum absorption for the base concerned. The mixture of base and HA was kept within the cuvette at 37°C and occasionally the optical density (O.D.) was measured. The sample was diluted to give an initial O.D. of about 1·2.

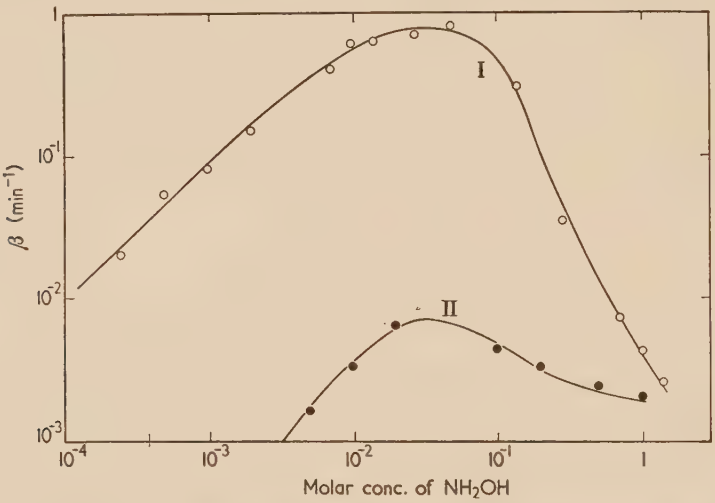

FIG. 1. The dependence of the rate of phage T4 inactivation (β) on the concentration of hydroxylamine.
Curve I: NH_2OH,HCl in buffer adjusted to pH 7·5 by NaOH.
Curve II: NH_2OH,HCl in buffer adjusted to pH 7·5 and sufficient NaCl added to make the total Na^+ concentration 1·3 M.

3. Results

(a) *The inactivation of phages by hydroxylamine*

In a mixture made by dissolving the HA† salt NH_2OH,HCl at various concentrations in phosphate buffer and adjusting the pH to 7·5 with NaOH, one finds that phages are inactivated approximately exponentially. In contrast to most chemical reactions, however, the rate β of phage T4 inactivation does not merely increase or reach a constant value with increasing concentration of NH_2OH,HCl, but instead

† Abbreviations used in this paper: HA = hydroxylamine, A = adenine, C = cytosine, G = guanine, T = thymine.

is maximal at about 0·03 M-NH_2OH,HCl, and for higher concentrations drops again by a factor of 300. This can be seen in Fig. 1 curve I. β is defined by the initial rate of change of ln B_0/B with time t (in min). "B" and "B_0" are the phage titers after and before HA treatment, respectively, as determined by plating on bacteria B.

These results show that the action of HA on phage T4 is inhibited by high concentrations of NH_2OH,HCl. The inhibition could be caused either by a high concentration of a non-reactive state of NH_2OH itself or by the high concentration of ions, i.e. Na^+ or Cl^-. (About 90% of the HCl in the HA salt has to be neutralized to give a pH of 7·5. The concentration of Na^+, therefore, is 0·9 times that of Cl^- plus about 0·1 M for the phosphate buffer.)

(i) *The protecting effect of ions*

In order to examine the effect of ions we inactivated phages in a mixture of buffer plus 0·02 M-NH_2OH,HCl plus various concentrations of NaCl adjusted to pH 7·5 by NaOH. The results revealed a striking protection against phage inactivation by high concentrations of NaCl, as shown in Table 1.

TABLE 1

Rate β of phage T4 inactivation by 0·02 M-hydroxylamine for different concentrations of NaCl

Concentration of NaCl in M	Rate β (min^{-1}) of inactivation
2·3	$5{\cdot}1 \times 10^{-3}$
1·3	$1{\cdot}8 \times 10^{-2}$
0·5	$3{\cdot}6 \times 10^{-2}$
0·1	$2{\cdot}1 \times 10^{-1}$
0·02	$5{\cdot}4 \times 10^{-1}$

(ii) *The protecting effect of hydroxylamine*

In addition to NaCl higher concentrations of hydroxylamine itself also inhibit its inactivating effect on phage T4. To show this we used various concentrations of NH_2OH,HCl in buffer, adjusted to pH 7·5 by NaOH, and added sufficient NaCl to make a total of about 1·3 M-NaCl. The result is plotted in Fig. 1 curve II.

(iii) *The inactivation curves of phage T4*

The treatment of T4 phages by HA does not produce perfectly exponential inactivation curves, but the rate β of inactivation increases with time. At high concentrations of HA this increase is noteworthy only after many hours of treatment, while at low concentrations the change in rate is quite rapid. Examples of this change in slope can be seen in Fig. 2. This figure depicts only a portion of the experimental range which stretches to 50 hr in time and to 10^{-7} in survival. This change of slope may be caused either by a decrease in the concentration of HA or by some property of the phage itself.

The concentration of HA decreases owing to a self-reaction of HA giving NH_3, N_2, and H_2O. The rate of this decomposition increases rapidly with the pH. At pH 7·5 the

HA concentration is reasonably stable, for the rate with which various cystosine bases react with HA is approximately constant for several hours (see Fig. 5). The slow increase of the inactivation rate of T4 phages at high HA concentration can be attributed, at least in part, to the slow decrease of HA concentration diminishing its own protective effect, as outlined in (ii).

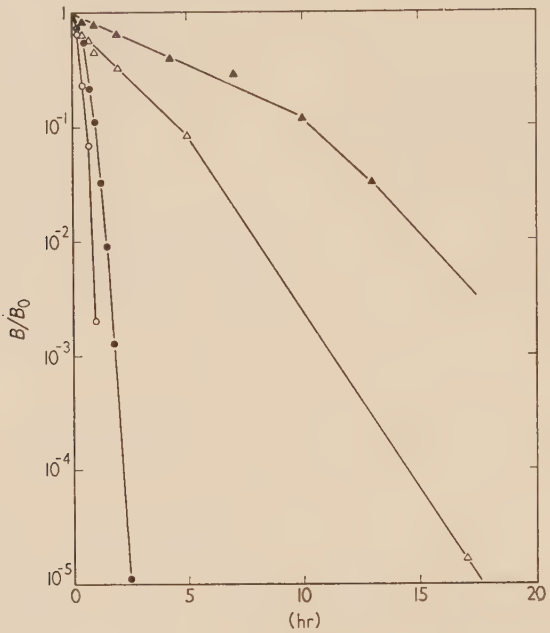

FIG. 2. Examples of the time course of phage T4 inactivation by various concentrations of NH_2OH and NaCl.

▲ = 0·2 M-NH_2OH + 1·2 M-NaCl + buffer
△ = 0·7 M-NH_2OH + 0·7 M-NaCl + buffer
● = 5×10^{-3} M-NH_2OH + 1·2 M-NaCl + buffer
○ = $2{\cdot}5 \times 10^{-4}$ M-NH_2OH + no NaCl + buffer

At low HA concentrations, however, the rapid increase of the inactivation rate cannot be explained in this way, for the protective effect of HA is no longer active (see Fig. 1), and hence a decrease in concentration should rather cause a decrease in the inactivation rate. Therefore this increase of rate must be due to some property of the phage itself (e.g. multiple-hit killing). When one plots the logarithm of $\frac{1}{t} \ln B_0/B$ against time one obtains a straight line. Extrapolation gives the inactivation rate β at zero time. It is this value that has been inserted in Fig. 1.

(iv) *A comparison of T4 and λ phages*

In 1 M-NH_2OH + 1·8 M-NaCl at pH 7·5 and 37°C, T4 and λ phages are inactivated at about the same rate.

(b) *The mutagenic effect of hydroxylamine*

At low concentrations of both HA and NaCl, T4 phages are rapidly inactivated without any appreciable increase in the frequency of *r*-type mutants. When the treatment employs high salt concentrations, however, the phages die slowly and HA

does induce mutations. The mutagenic effect is especially pronounced when both the salt and the HA concentrations are high, which keeps the rate of inactivation at a minimum. The dependence of the r mutation frequency on the concentration of HA and on the survival can be seen in Fig. 3. For small inactivation the frequency of r plaques plus mottled plaques increases linearly with time, or with lethal hits ($=\ln B_0/B$); this shows

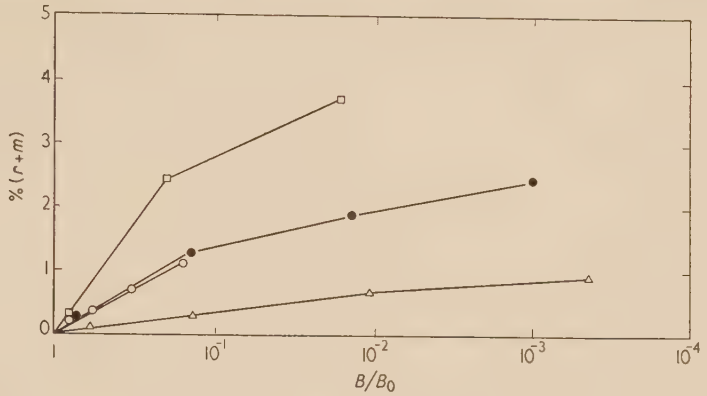

FIG. 3. Increase of r plus mottled plaques with respect to survivors of hydroxylamine inactivation.
□ = 1·0 M-NH_2OH + 1·3 M-NaCl
● = 0·5 M-NH_2OH + 1·3 M-NaCl
○ = 1·0 M-NH_2OH + 1·0 M-NaCl
△ = 0·2 M-NH_2OH + 1·3 M-NaCl

that mutation induction is a one-hit phenomenon (in contrast to a two- (or more) hit phenomenon, which would give a quadratic, or higher, increase). For longer times of treatment the slope of the curve decreases; this corresponds to the above-mentioned increase of the inactivation rate with time which is apparently caused by an increasing fraction of the non-mutagenic inactivation. Figure 3 also shows how the frequency of r plaques plus mottled plaques per lethal hit increases with the concentration of HA.

It is clear from these results that HA can exert two chemically different effects on phage T4: (1) A rapid action which merely inactivates phages without being mutagenic; it is inhibited by high ion and NH_2OH concentrations. (2) A slow action which is mutagenic and not subject to this kind of inhibition. This mutagenic effect can show up in full strength only at high concentrations of ions (NaCl) and NH_2OH since otherwise it is masked by the rapid inactivation. The slope β of the phage inactivation curve is then composed of the sum of the rate β_r with which the rapid inactivation takes place and the rate β_m for the slow mutagenic effect:

$$\frac{B}{B_0} = e^{-\beta t} = e^{-(\beta_r + \beta_m)t} \qquad (1)$$

(i) *The frequency of mottled versus* r *plaques*

Each mutagenic hit within an r region of phage T4 causes the appearance of either an r or a mottled plaque, unless the DNA (or phage chromosomal) strand in which the hit occurred is unable to produce live progeny for some other reason (e.g. a second hit). If we neglect the background of spontaneous mutants the frequency, $r + m$, of r and mottled plaques per viable phage should increase according to the equation

$$r + m = \gamma t \qquad (2)$$

at least for a small number of lethal hits. Initially all of the induced r mutations

should give rise to mottled plaques. For longer mutagenic treatment, however, the likelihood increases that a second hit either prevents the other strand of the phage chromosome from producing viable phages or induces an r mutation in that strand too. The frequency, m, of mottled plaques per viable phage should therefore be modulated by an exponentially decreasing function:

$$m = \gamma t e^{-p\beta_m t} \tag{3}$$

Here p is the fraction of hits (measured in units of lethal hits, $\beta_m t$, of the mutagenic type) that prevent one chromosomal strand from producing a viable r^+ phage. Since HA exerts two inactivating effects (β_r and β_m) we cannot measure directly the number, $\beta_m t$, of mutagenic hits. It is possible, however, to obtain a relation which is independent of the non-mutagenic inactivation by inserting (2) into (3). We then get

$$\frac{m}{r+m} = e^{-p\frac{\beta_m}{\gamma}(r+m)} \tag{4}$$

The exponent should also be independent of the concentration of HA since both β_m and γ should increase linearly with this concentration.

We have examined this relationship (4) by determining the frequency of both r and mottled plaques after different times of HA treatment and under various conditions. The result is shown in Fig. 4. The assumption of a negligible background of

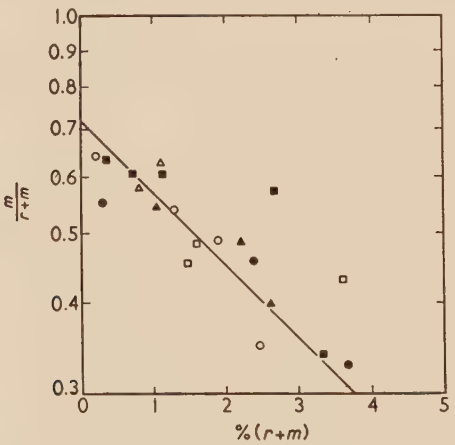

Fig. 4. Decrease in the fraction of mottled plaques with increasing mutational hits. Points indicated by different symbols belong to independent experiments.

m = frequency of mottled plaques per plaque-forming phage
r = frequency of r plaques per plaque-forming phage

spontaneous mutants is valid for all points in this figure. Although the individual values of $\frac{m}{r+m}$ scatter considerably, this ratio clearly decreases slowly and apparently exponentially. The value of the exponent, $p\frac{\beta_m}{\gamma}$, is about 0·23. If we approximate the r mutation frequency per lethal mutagenic hit, $\frac{\gamma}{\beta_m}$, by the experimental $\frac{\gamma}{\beta}$ value obtained for the highest concentration of both NaCl and HA we get from Fig. 3 $\frac{\gamma}{\beta_m} \approx \left(\frac{\gamma}{\beta}\right) = 0{\cdot}015$. It follows that:

$$p = 0{\cdot}23\frac{\gamma}{\beta_m} \approx 3{\cdot}5 \times 10^{-2}$$

The decrease of $\frac{m}{r+m}$ can also be observed with respect to increasing lethal hits, but in that case it is slower the smaller the concentration of HA or salt, as expected from the aforementioned concentration effect on phage inactivation.

It should be noted that the extrapolation of the $\frac{m}{r+m}$ curve back to zero frequency of $(r + m)$ does not go to 1, as one would have expected, but to about 0·7. In one experiment we have isolated the phages from 125 plaques which had been scored according to r and m plaques giving $\frac{m}{r+m} = 0{\cdot}60$. When these phages were plated on B a few of the original plaques denoted r also gave rise to both r and some wild type plaques. This could be due either to contamination by a wild type plaque which was adjacent to the original r plaque or to some standard type phages in an apparent "r" plaque which should have been scored as "mottled". If we assume, as an upper estimate, all plaques to be mottled which gave 2 kinds of plaques on replating we get $\frac{m}{r+m} = 0{\cdot}71$. Hence the straight line in Fig. 4 may be slightly shifted upwards owing to such scoring mistakes but nevertheless the extrapolation of $\frac{m}{r+m}$ to zero treatment would not give 1 but about 0·8.

(ii) *The genetic distribution of NH_2OH-induced* r*II-type mutants*

We isolated 36 *r*II-type mutants induced by NH_2OH under conditions in which about 2% of the mutants could be spontaneous. These mutants have been mapped genetically, using the methods described by Benzer & Freese (1958); the result is summarized in Table 2. The mutations are distributed over the whole *r*II region and some of them seem to coincide (no recombination between them observed).

Table 2

Genetic distribution of r*-type mutants induced by treatment of standard phage T4 with 1* M*-hydroxylamine + 1* M*-NaCl, pH 7·5 at 37°C for 18 hr*

No. of mutants at same site	1	2	3	4
No. of such sites observed	19	7	1	0

(iii) *The reversion properties of the induced mutants*

Most of these HA-induced mutants revert spontaneously at a very low rate. They have been tested for the induction of reverse mutations by 2-aminopurine and 5-bromodeoxyuridine (for method see Freese, 1959). Thirty-four of the 36 mutants, i.e. 94%, showed reversion inducibility by 2-aminopurine and 22 mutants, i.e. 61%, by 5-bromodeoxyuridine. Only two mutants gave no response with either agent; both have a high spontaneous reversion frequency.

(iv) *The effect of* N-*methylhydroxylamine*

In the attempt to separate the rapid and the mutagenic inactivation of phage T4 we prepared N-methylhydroxylamine (MeHA) as the HCl salt by the method of Beckmann (1909). When this substance is neutralized and used in the same way as

TABLE 3

Rate β of phage T4 inactivation by various concentrations of N-*methylhydroxylamine, CH_3NHOH, HCl, in buffer, adjusted to pH 7·5 by NaOH*

CH_3NHOH, HCl	10^{-4}M	10^{-3}M	10^{-2}M	10^{-1}M	1M
β in min^{-1}	$7{\cdot}7 \times 10^{-2}$	$7{\cdot}7 \times 10^{-1}$	$3{\cdot}5 \times 10^{-2}$	$2{\cdot}7 \times 10^{-2}$	1×10^{-3}

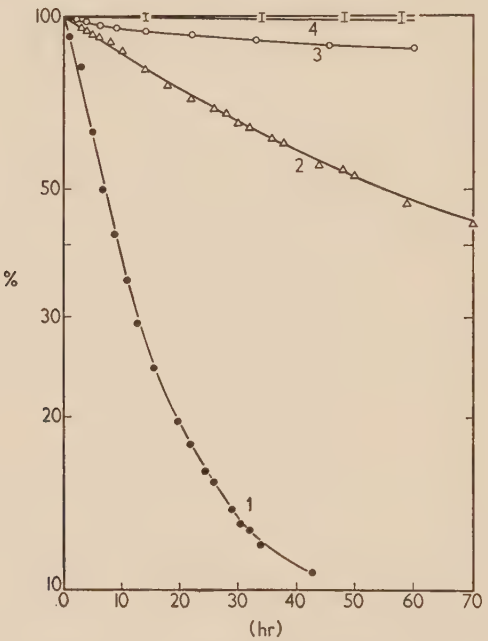

FIG. 5. Decrease of u.v. absorption of nucleic acid bases and deoxynucleosides during treatment with 1 M-hydroxylamine, pH 7·5 at 37°C.

1 = cytosine
2 = deoxycytidine
3 = hydroxymethylcytosine
No reaction observed with:
4 = adenine, guanine, thymine, methylcytosine, adeninedeoxyriboside, guaninedeoxyriboside, thyminedoexyriboside. Most O.D. measurements gave values which are located in the area given by two lines and sign I shows the intervals in which some extreme values are scattered.

HA before, one observes a similar concentration dependence of the phage T4 inactivation as for HA. The initial slopes β are given in Table 3 for different concentrations of CH_3NHOH, HCl. In contrast to HA, however, we could not detect any significant mutagenic effect, even at high concentrations of both MeHA (1 M) and NaCl (1·8 M),

either in the forward direction ($r^+ \rightarrow r$) or in the reverse direction ($r\text{II} \rightarrow r^+$), for which e.g. the mutant *r* AP 72 is highly reversion-inducible by HA but did not show any effect for MeHA.

MeHA is apparently quite similar to HA with respect to the rapid inactivation of phages, but it differs from HA by not showing any significant mutagenic effect.

(c) *The reaction of hydroxylamine with the bases occurring in nucleic acids*

The only DNA bases for which we could detect a chemical reaction with HA are cytosine (C) and 5-hydroxymethylcytosine (HMC). This reaction can easily be observed since the chemically altered cytosine no longer absorbs u.v. in the wavelength range near 260 mμ. A quantitative measurement of the decrease of O.D. (read at the wavelength of maximum absorption of each base) is shown in Fig. 5. The rate of base destruction decreases very slowly owing to the self-extinction of HA mentioned earlier. The free base C reacts 4 times faster than its deoxynucleoside. HMC, the base contained in phage T4, reacts about 10 times slower than does cytosine. No change in u.v. absorption could be observed for T, A, G, or their deoxynucleosides, or for 5-methylcytosine (MC). The bases T, A, G, and their deoxynucleosides were also treated for 24 hr at 37°C with 1 M-HA at pH 7·5 and the mixture added to a column (Dowex 1, formate form, mixture added at pH 12 and eluted with 0·2 M-ammonium formate at pH 9·6, then pH 9·0, and finally 0·5 M-formic acid). No new u.v.-absorbing peak could be observed when 5 mg of the base were used. The reaction of HA with these bases, of which T is the most likely to be attacked, must be very small. At present we can only say that the reaction with C is at least 30 times and that with HMC at least 5 times faster than that with MC, T, A or G. The products of these reactions are under investigation.

For the RNA bases the decrease in u.v. absorption can be observed for both uracil and cytosine; uridine reacts about twice as fast as cytidine. Pseudouridine, however, did not show any decrease of u.v. absorption within 9 hr of treatment by 1 M-HA.

4. Discussion

Hydroxylamine (HA) exerts two chemically different effects on phage T4, a rapid non-mutagenic and a slow highly mutagenic action.

(1) *The rapid inactivation* of T2 and T4 phages by lower concentrations of hydroxylamine (HA) has been described already by Kozloff, Lute & Henderson (1957). By analogy with the action of other chemicals these authors postulated that HA breaks thiol-ester bonds by which the phage tail fibers are attached to the tail spike. We observed that high concentrations of NaCl and even of HA itself strongly inhibit this action of HA. While the breakage of thiol-ester bonds is probably caused either by the neutral NH_2OH or by the negatively charged $NH{\cdot}OH^-$, the protecting effect of HA probably arises by the attachment of another non-reactive HA molecule, e.g. NH_3OH^+ or NH_2OH, to the sensitive site of the phage.

(2) *The inactivation* of several RNA viruses, or their isolated "RNA", has been observed by Franklin & Wecker (1959). They assumed that HA inactivates by separating the RNA from a covalently bound peptide. The speed with which the inactivation took place may suggest that it is mainly due to the breakage of such an

ester bond; nevertheless, at least some of the inactivation must be caused by the reaction of HA with RNA itself since HA attacks both cytosine and uracil.

(3) *The strong mutagenic effect* of HA on phage T4 is observed only under conditions where the rapid non-mutagenic inactivation is practically completely inhibited by high concentrations of ions and HA. The frequency of mutants per viable phage at first increases linearly with respect to time or lethal hits, indicating that these mutations are induced by the alteration of a single base in DNA. For longer treatment the curve levels off, probably because HA is slowly used up by its self-annihilation.

(4) For very small inactivation times most of the *r* mutants appear as *mottled plaques*. The same observation has been made for the induction of mutations by all other mutagenic agents and it has been measured quantitatively by Pratt & Stent (1959) in the case of 5-bromouracil, and by Tessman (1959) for nitrous acid-induced mutants. The mottled plaques apparently reflect the dual nature of the information carried by DNA and the fact that only one base in a nucleotide pair is altered by the mutagenic agent. Upon duplication one normal and one altered DNA are produced. Whether this altered DNA can still throw out non-mutated DNA, or whether it is now completely mutant, has still to be examined.

With increasing number of lethal hits ($\beta_m t$), of the mutagenic type, more and more of the induced *r* mutants appear as pure *r* plaques. This is apparently due to a second hit in the complementary DNA strand or in that part of the phage chromosome that stays together with it in the course of phage multiplication. This second hit may either prevent the other chromosomal strand from producing any viable phage or it may cause a pure *r* plaque by inducing a second *r* mutation in that second strand too. From the slope in Fig. 4 one can conclude that the frequency of such a second hit is about $p = 0{\cdot}035$ of the mutagenic lethal hits ($\beta_m t$). This frequency is only about three times larger than that with which *r* mutations are induced (see Fig. 3).

It is interesting that the curve in Fig. 4 does not extrapolate back to $\frac{m}{r+m} = 1$ for zero lethal hits but rather to 0·7 to 0·8. This suggests that, even without induction of mutations, about 35 to 50% of the phages lose one of their two information strands during phage duplication. (If q is the probability that a given DNA strand does *not* get lost, e.g. the wild type one in a chemically altered DNA, then $q = \left(\frac{m}{m+r}\right)_0$. The probability that neither of the two complementary DNA strands gets lost is q^2, and that at least one of them gets lost is $1 - q^2$.)

(5) There are *many genetic sites* which can be mutated by HA, as is shown by the distribution of HA induced mutants within the *r*II region (Table 2). About 94% of the induced mutants are apparently due to base-pair transitions (Freese 1959) since they can be induced to revert by at least one of the base analogues 2-aminopurine and 5-bromodeoxyuridine. The induction of reverse mutations by HA will be discussed in another paper.

(6) *The chemical reaction of HA* with the DNA bases seems to be due to its tendency to attack electrophilic groups. We have not noticed any effect on the free purines or their deoxynucleosides, on thymine or deoxythymidine, or on 5-methylcytosine under our mutagenic conditions (pH 7·5 and 37°C). Cytosine and deoxycytidine, in contrast, react quite extensively (see Fig. 5). The result of this reaction is the destruction of the resonance and hence the u.v. absorption of the unsaturated pyrimidine ring. The detailed reaction mechanism is under investigation.

(7) The much stronger reactivity of HA with cytosine than with thymine suggests that this agent can be used to induce base-pair transitions preferentially in one direction, i.e.

$$\begin{matrix} C & & T \\ G & \longrightarrow & A \end{matrix}$$

It is furthermore significant that HA reacts more rapidly with cytosine than with 5-methyl- or 5-hydroxymethyl-cytosine. The directional effect of HA-induced base-pair transitions should therefore be even more pronounced for a cytosine than for a hydroxymethylcytosine containing DNA.

The stronger effect of HA on cytosine-containing DNA is indicated by a comparison of T4 and λ phages. While u.v. inactivates T4 about 7 times more rapidly than λ (Weigle & Delbrück, 1951; Luria, 1947) HA inactivates them both at the same rate (at high concentrations of ions and HA).

(8) Especially interesting should be the effect of HA for those systems which contain in their DNA two kinds of cytosine bases having different substituents at the 5-position. Such bases apparently do not replace each other randomly; instead each prefers being next to certain nucleotides. For example it has been demonstrated for DNA containing both cytosine and 5-methylcytosine (Sinsheimer, 1955) that methylcytosine is found especially frequently next to guanine. If HA attacks one of the two bases much more frequently than the other (as for the combination C and MC, or for C and HMC, etc.) mutagenic base-pair changes may be induced preferentially next to certain bases which can be determined by a chemical nearest-neighbor analysis.

(9) The much higher reactivity of uracil and uridine than of pseudouridine with HA can be used to distinguish between these compounds.

REFERENCES

Beckmann, E. (1909). *Liebigs Ann.* **365**, 204.
Benzer, S. & Freese, E. (1958). *Proc. Nat. Acad. Sci., Wash.* **44**, 112.
Brookes, P. & Lawley, P. D. (1960). *J. Chem. Soc.* **112**, 539.
Franklin, R. M. & Wecker, E. (1959). *Nature*, **184**, 343.
Freese, E. (1959). *Proc. Nat. Acad. Sci., Wash.* **45**, 622.
Kozloff, L. M., Lute, M. & Henderson, K. (1957). *J. Biol. Chem.* **228**, 511.
Luria, S. A. (1947). *Proc. Nat. Acad. Sci., Wash.* **33**, 253.
Pratt, D. & Stent, G. S. (1959). *Proc. Nat. Acad. Sci., Wash.* **45**, 1507.
Reiner, B. & Zamenhof, S. (1957). *J. Biol. Chem.* **228**, 475.
Schuster, H. & Schramm, G. (1958). *Z. Naturf.* **13***b*, 697.
Sinsheimer, R. L. (1955). *J. Biol. Chem.* **215**, 579.
Stacey, K. A., Cobb, M., Consens, S. F. & Alexander, P. (1958). *Ann. N.Y. Acad. Sci.* **68**, 682.
Staehlin, M. (1958). *Biochim. biophys. Acta*, **31**, 448.
Takemura, S. (1959). *Bull. Chem. Soc., Japan* **32**, 920.
Tamm, C., Hodes, M. E. & Chargaff, E. (1952). *J. Biol. Chem.* **195**, 45.
Tessman, I. (1959). *Virology*, **9**, 375.
Weigle, J. J. & Delbrück, M. (1951). *J. Bact.* **62**, 301.

J. Mol. Biol. (1961) **3**, 144–155

Thymine Deficiency and the Normal DNA Replication Cycle. I

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In the absence of thymine, bacterial strains that require this compound for growth lose viability in a characteristic manner. If, at the time when thymine is removed from a culture, protein and RNA synthesis are inhibited a small fraction of the cells is found to be immune to thymineless death. Under conditions of inhibited protein and RNA synthesis total DNA is found to increase by 40% or more. During this period of DNA synthesis the entire cell population gradually becomes immune; i.e. reaches a state in which subsequent removal of thymine does not cause death. To account for these observations we postulate that protein and/or RNA synthesis is necessary to *initiate* but not to *sustain* DNA replication.

During renewed growth the acquired immunity to thymineless death is gradually lost and, in parallel, DNA synthesis is resumed. The steps by which a new DNA replication cycle is initiated require protein and/or RNA synthesis but take place as efficiently in the presence as in the absence of thymine. The chemical nature of these steps is unknown, but they are clearly independent of the replication process proper.

1. Introduction

The effect of thymine deficiency on viability and on the synthetic activities in thymine-requiring strains of *Escherichia coli* was first described by Cohen & Barner (1954). It was found that removal of thymine immediately stopped DNA synthesis without initially affecting protein and RNA synthesis. Under these conditions a pronounced loss of viability was observed. If all three synthetic activities were simultaneously blocked the loss of viability was somewhat reduced. We have extended such studies, concentrating on the effect of thymine-starvation in cells synthesizing little or no protein and RNA. Our results provide new information about the normal DNA replication cycle.

In bacteria, DNA synthesis is regularly found to continue for some time after protein synthesis has been inhibited. This inhibition can be effected by transferring cells growing in a rich medium to minimal medium (Kjeldgaard, Maaløe & Schaechter, 1958), by withdrawing a required amino acid from the medium (Goldstein, Goldstein, Brown & Chou, 1959) or by adding chloramphenicol (see, e.g. Billen, 1959). In experiments of this kind total DNA increases by no less than 40% and sometimes more.

To account for this increase we assume (a) that the DNA replication cycle, once initiated in a cell, runs to completion independently of protein and RNA synthesis, and (b) that a new round of replication cannot be initiated as long as inhibition is maintained. Thus we postulate a step between successive rounds of DNA replication,

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which requires protein and/or RNA synthesis. This step must be of short duration since DNA synthesis is known to be continuous throughout most of the division cycle (Schaechter, Bentzon & Maaløe, 1959; van Tubergen, personal communication).

For a case in which protein and RNA synthesis is imagined to be completely and instantaneously inhibited our theory predicts a DNA increment very close to 40% (see Discussion). This agrees with the minimum value observed experimentally. If inhibition is assumed to be incomplete, or to be established gradually, larger DNA increments are predicted.

Selective inhibition of DNA, *or* of protein and RNA synthesis, can readily be achieved by using a triple auxotroph of *E. coli* requiring thymine (T), arginine (A) and uracil (U). This strain which has been extensively studied by Kanazir, Barner, Flaks & Cohen (1959) was used in our experiments to provide evidence that the DNA synthesis which occurs after withdrawal of A and U represents the completion of already initiated replication.

2. Materials and Methods

(a) *Bacterial strains and growth conditions*

The thymine-requiring bacterium *E. coli* $15T^-$ and the triple auxotroph, *E. coli* $15T^-A^-U^-$ (requiring thymine, arginine and uracil) were obtained through the courtesy of Professor S. S. Cohen. We have found that the uracil requirement of this strain is satisfied equally well by uracil and by cytosine. Another double mutant of *E. coli* $15T^-$ (requiring proline and tryptophan in addition to thymine) was kindly furnished by Dr. D. Luzzati.

The bacteria were grown aerobically at 37°C in a synthetic medium (pH 7·4) containing in one liter of water: 12 g 2-amino-2-hydroxymethylpropane-1:3-diol (tris), 2 g KCl, 2 g NH_4Cl, 0·5 g $MgCl_2,6H_2O$, 0·05 g $Na_2HPO_4,2H_2O$, 0·02 g Na_2SO_4, 7·5 ml. conc. HCl and 0·5% glucose as carbon source. Supplements of thymine (2 μg/ml.), uracil (10 μg/ml.) and arginine (20 μg/ml.) were added when required.

Growth (net mass increase) was followed by optical density readings (450 mμ, 1 cm path length) against a tris buffer blank in a Zeiss PMQ II spectrophotometer. The optical density was maintained between 0·10 and 0·50 (i.e. between approximately $0{\cdot}8 \times 10^8$ and $4{\cdot}0 \times 10^8$ cells/ml.) by periodic dilution in prewarmed medium. Exponential growth was maintained for at least three generations before the beginning of an experiment.

Viable cell counts were determined by the agar layer technique. Dilution was made in tris buffer (tris medium without glucose) at room temperature and portions containing 300 to 600 viable cells each were mixed with 3 ml. portions of 45°C tris agar (0·8% agar) containing thymine, arginine and uracil. This mixture was poured over a 5 mm layer of tris medium with 1% agar and incubated at 37°C.

To change growth media or remove supplements the bacteria were harvested by suction filtration on a coarse-porosity collodion filter (Membranfilter, Göttingen, Germany) and were rinsed on the filter with several volumes of 37°C tris buffer before being resuspended in fresh, prewarmed medium. It took less than 2 min to complete the process of filtration and resuspension. No loss in viability due to the filtering procedure could be demonstrated.

(b) *Isotope incorporation and radioactivity assay*

^{14}C-thymine (9·5 mc/m-mole) was obtained from the Radiochemical Centre, Amersham, England and used at a concentration of 0·0159 μmole/ml. in growth medium for following DNA synthesis.

For assay of radioactivity incorporated into DNA, 1 ml. portions from the culture were added to tubes containing 5 ml. ice-cold 5% trichloroacetic acid. Subsequently, 2 ml. samples were collected on (27 mm ⌀) collodion filters by suction filtration and rinsed twice with 5 ml. of distilled water. The filters were glued to aluminium planchets for counting with a thin-window GM-counter.

3. Experimental

(a) *General observations on thymineless death in strain 15T⁻*

The effect of growth rate on thymineless death was examined by following viability and net mass synthesis in thymineless cultures in two different media. Medium I was the tris synthetic medium already described; medium II contained in addition 1·5% Difco casamino acids. The generation times in these media were 50 min and 35 min respectively. A culture in exponential growth at 37 °C in medium I was filtered and portions resuspended in prewarmed I and II media, both without thymine. After an initial lag period of 30 min, loss of viability proceeded exponentially and at similar rates in the two cultures (Fig. 1(a)). Mass synthesis began at the rates characteristic of growth in the respective media, and the final increment was considerably greater in the more rapidly growing culture (Fig. 2(a)). The fact that thymineless death occurs at the same rate in the two media shows that the processes resulting in loss of viability are more or less independent of the rate of cytoplasmic synthesis. It would seem natural then to associate the "killing event" with the process of DNA synthesis, and our observations do not contradict such an assumption since it has been found that the low pre-shift rate of DNA synthesis is maintained for some time after shifting a culture from a minimal to an enriched medium (Kjeldgaard *et al.*, 1958).

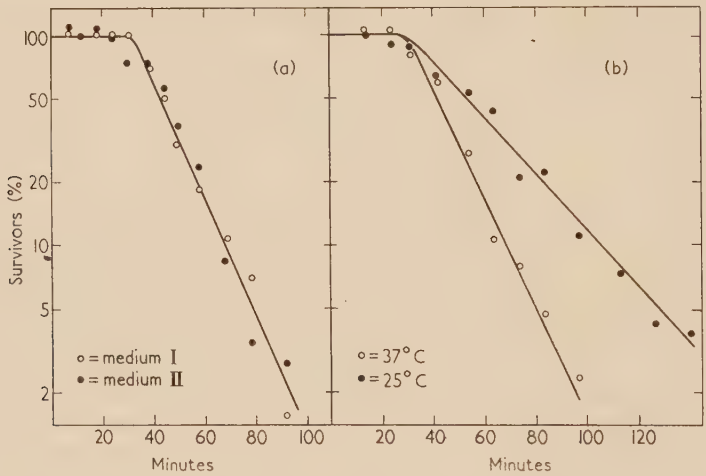

Fig. 1(a). Loss of viability during thymineless growth of *E. coli*, strain 15T⁻. Following balanced growth in a glucose-salts medium with thymine, cells were washed and samples transferred to thymine-free medium, without (I) and with amino acids added (II).

(b) As (a), except that, after transfer, the two cell samples were held in medium I at 37°C and at 25°C respectively.

When the growth rate was varied by changing the incubation temperature a different pattern was observed. Bacteria growing in medium I at 37 °C were filtered and resuspended in the *same* medium without thymine at 37 °C and at 25 °C, respectively. As expected the initial rate of mass synthesis at 37 °C was about twice that at 25 °C; however, the final mass increments were identical (Fig. 2(b)). The loss of viability was exponential in both cases and showed the *same* temperature dependence as did the rate of mass synthesis (Fig. 1(b)). A culture kept at 25 °C throughout the

experiment exhibited a 45 min rather than a 30 min lag before the onset of thymineless death, which then proceeded at the characteristic 25°C rate.

Samples from a thymineless culture at 37°C were stained with acridine orange and observed by fluorescence microscopy at different stages in thymineless growth. The nuclear areas were visible in these preparations as yellow patches in the red-stained cells (Anderson, Armstrong & Niven, 1959). In confirmation of the observations of

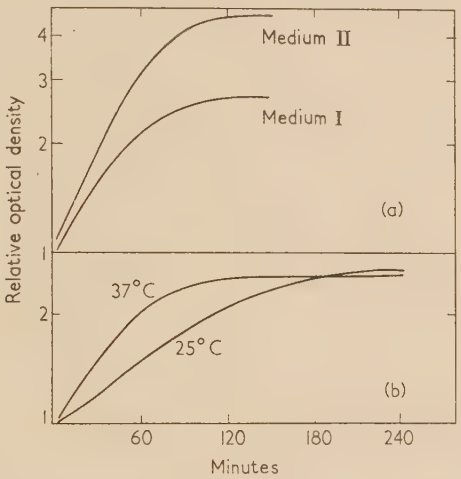

FIG. 2(a) and (b). Relative increase in optical density (O.D.) in the four cell samples represented by the curves of Fig. 1(a) and (b).

Cohen & Barner (1954), and Hanawalt (1958), the cells were observed to elongate but not to divide. Within an hour, dark (unstained) patches were seen in the red-staining areas of the filaments. On prolonged incubation these "empty areas" increased in number.

^{32}P, incorporated from $^{32}PO_4$ by an exponentially growing culture of *E. coli*, 15T$^-$, was not lost from the cells over a 4 hr period of thymineless growth. Thus no appreciable breakdown and release of DNA fragments into the medium had occurred at a time when survival was less than 10^{-4} and mass synthesis had ceased.

(b) *Thymineless death during low-level RNA and protein synthesis*

Barner & Cohen (1957) have shown that thymineless death is reduced in an amino-acid requiring auxotroph of a thymineless bacterium in the absence of the amino acid, although protein synthesis is not totally inhibited. Similarly, in a U-requiring strain, thymineless death is reduced in the absence of U; appreciable residual protein synthesis was observed in this case and attributed to an active RNA turnover (Barner & Cohen, 1958). A much lower residual level of both RNA and protein synthesis is obtained by removing both A and U from a culture of the triple mutant, *E. coli* 15T$^-$ A$^-$U$^-$. This strain was therefore used in our experiments. For convenience a thymineless medium containing both A and U will be designated (—T, + AU), and analogously for other combinations of T, A and U.

An exponentially growing culture at 37°C of the triple mutant behaved exactly as the parent strain, *E. coli* 15T$^-$, when transferred to a (— T, + AU) medium (Curve I, Fig. 3). After the initial 30 min lag period loss of viability proceeded exponentially,

with a half-life of about 10 min, for at least 4 decades. In a (— T, — AU) medium the lag was unchanged, the half-life was increased somewhat, but thymineless death stopped almost completely at a survival of about 3%. A similar experiment at 25°C showed 10% survival. These results show that, in the majority of the cells, the course of thymineless death is nearly the same whether or not protein synthesis is inhibited. The cells which are immune to thymineless death in the (— T, — AU) medium may therefore represent a particular state in the bacterial growth cycle.

The conclusion that protein synthesis, rather than a specific requirement for arginine, is involved in selecting the class of immune cells was checked in several ways. Thus in a (— T, + A, — U) medium containing 10 μg/ml. chloramphenicol a 6% survival level was obtained, and a 3% survival level was found when all three requirements were withheld from the triple-mutant which in addition to thymine requires proline and tryptophan. Slight differences in the surviving fraction and in the sensitivity of that fraction to further decay could be attributed to different degrees of "leakiness" in experiments where protein synthesis was inhibited by different means.

Cells that undergo thymineless death in the (— T, — AU) medium (but *not* in the (— T, + AU) medium, see p. 147) might liberate thymine in one form or another. It is conceivable that cells which have not yet suffered damage might be rescued by assimilating thymine liberated from other cells. This trivial explanation was eliminated by showing that the course and extent of thymineless death was the same in fresh medium and in a sterile filtrate of (— T, — AU) medium, in which cells at 4 times the usual density had been incubated for 2·5 hr at 37°C.

(c) *Effect of incubation in (+ T, — AU) medium*

It was suggested above that the small fraction of cells immune to thymineless death in the (— T, — AU) medium might represent a particular phase in the division cycle. This interpretation was tested in experiments of the following type: A and U were removed from an exponentially growing culture, and after various periods of time T was also removed and the fraction of the population immune to thymineless death determined by colony count. As shown in Fig. 3 (Curves II to V), pre-incubation *with* thymine gradually brings the entire population into the immune state. Complete immunity was regularly obtained after 90 minutes of inhibition of protein synthesis in the presence of T. The proline- and tryptophan-requiring strain, derived from *E. coli* strain 15T⁻, similarly develops full immunity after 90 minutes of inhibition of protein synthesis in a (+ T, — proline, — tryptophan) medium, as shown by Dr. D. Luzzati (personal communication) and by ourselves.

The rescue effect of pre-incubation with thymine under conditions which preclude net mass synthesis could also be demonstrated with strain 15T⁻. A culture in the glucose salts medium enriched with thymine and 19 amino acids was divided into two portions; one served as control and the other was filtered to remove the amino acids, thereby temporarily inhibiting protein and RNA synthesis (Kjeldgaard *et al.*, 1958). After 30 min thymine was also removed; and at the same time the amino acids as well as thymine were removed from the control culture. Under these conditions the lag before the commencement of thymineless death in the control was 30 min, against 75 min in the pre-incubated culture; the viable counts decreased exponentially at very similar rates in the two cultures. The onset of thymineless death in the pre-incubated culture coincided roughly with the time at which mass synthesis was beginning after

the shift to minimal medium. In other words the pretreatment caused immunity to thymineless death and this immunity lasted until the culture began to adapt to growth in the depleted medium. The same general picture was obtained when a broth culture of strain 15T⁻ was shifted to minimal medium with thymine and held for 30 min before removing thymine.

These results suggested to us that, during incubation *with* T, but in the absence of protein and RNA synthesis, all the cells gradually completed their DNA replication cycle. This would bring the cells into a state characterized by the absence of DNA synthesis, and thereby prevent thymineless death. To account for the persistance of immunity in a (— T, — AU) medium we assume that a step involving protein and/or RNA synthesis is necessary to initiate a new round of replication.

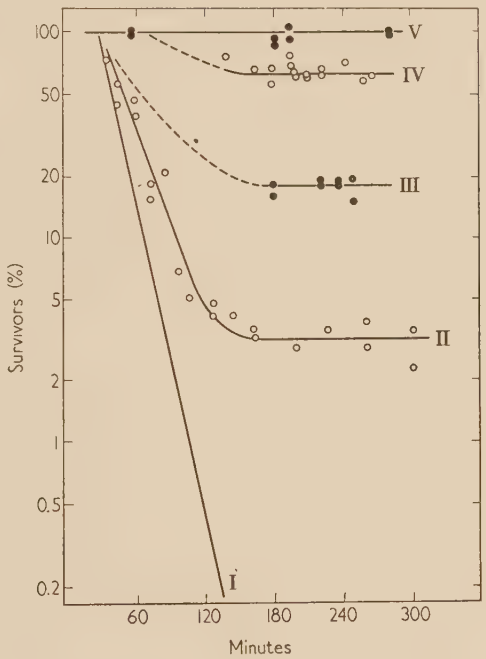

FIG. 3. The effect of pre-incubation in (+ T, — AU) medium for various times upon survival during subsequent incubation in (— T, — AU) medium. Curves II-V are compiled from several experiments, they correspond to 0, 30, 60, and 90 min of pre-incubation respectively; for comparison curve I is included which shows the course of thymineless death in a (— T, + AU) medium.

DNA synthesis after transfer to a (+ T, — AU) medium was examined by following the incorporation of ^{14}C-thymine. Fig. 4(a) shows that the rate of incorporation which initially equaled that of the control culture gradually decreased, and that by 90 min incorporation had stopped. During this period about 20% of the cells divided. In Fig. 4(b) the uptake of thymine by the control culture is plotted against the mass increment; from this linear plot the activity corresponding to full labeling at time zero was derived and compared to the activity attained after 90 min in the (+ T, — AU) medium. In this way, it was estimated that by 90 min DNA had increased by about 55%. The significance of this value and of the amount of cell division during the 90 min period will be discussed in the next paper of this issue.

(d) *Growth in complete medium following incubation in (+ T, — AU) medium*

When A and U are added back to a culture after incubation for 90 min in the (+ T, — AU) medium, the optical density at once begins to increase and after about 5 min exponential growth at the normal rate is re-established. We have examined, (a) the way in which the population returns to its normal sensitivity to thymineless death in the (— T, — AU) medium, and (b) the resumption of DNA synthesis and the return of the culture to its normal DNA/mass ratio. Figure 5 illustrates the changes in degree of immunity to thymineless death under various conditions: Curve A shows the increase in immunity during the 90 min period in the (+ T, — AU) medium (compare Fig. 3); curve B shows how the immunity attained during the 90 min is lost gradually during the first 50 minutes of growth in complete medium; curve C shows the more abrupt loss of immunity observed in the 3%-fraction naturally

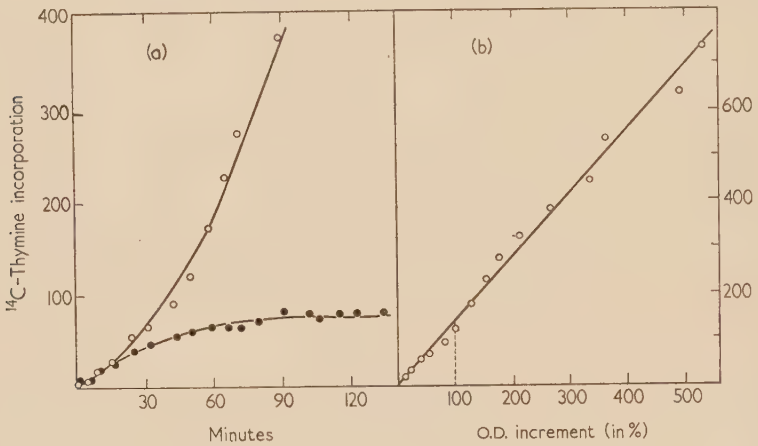

FIG. 4(a). Comparison of ^{14}C-thymine incorporation into trichloroacetic acid-insoluble material (DNA) during growth in (+ T, + AU) medium, upper curve; and in (+ T, — AU) medium, lower curve.

(b) Incorporated ^{14}C-activity plotted against O.D. increment expressed as percent of the initial O.D. value. From the activity corresponding to one doubling (140), and the maximum activity (75) obtained during incubation in (+ T, — AU) medium, it can be seen that, in this medium, total DNA increased by about 55%.

resistant to thymineless death in the (— T, — AU) medium. This last experiment was performed by first allowing the 3%-fraction to become established by incubating for 140 min in the (— T, — AU) medium; the experiment proper was then started by adding T, A, and U, and at the times indicated in Fig. 5 all three compounds were again withdrawn to test the susceptibility of the 3%-fraction to thymineless death.

We have also studied the effect on immunity of pre-incubation for 90 min in the (+ T, — AU) medium followed by growth in a (— T, + AU) medium. In this situation the lag before the onset of thymineless death is prolonged; during the first hour of incubation in the (— T, + AU) medium the increase in optical density equals that observed in complete medium, and no loss of viability occurs. In this period the cells grow in mass but no DNA is synthesized (see Fig. 7). Nevertheless, an increasing fraction of the cells are found to enter the state in which they will suffer thymineless death if tested in the (— T, — AU) medium. Figure 5 includes an observation which shows that the *loss* of immunity occurs at similar rates in (+ T, + AU) and in

(— T, + AU) media. From this we conclude that the steps necessary to bring a cell from the immune to the susceptible state require protein and/or RNA synthesis but are independent of DNA synthesis.

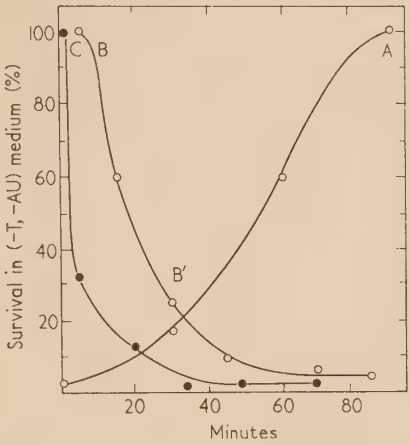

FIG. 5. All points show survival levels after incubation for 3 to 4 hr at 37°C in (— T, — AU) medium.

Curve A: Pre-incubation in (+ T, — AU) medium for times indicated on the abscissa; shows build up of immunity (cf. Fig. 3).

Curve B: 90 min in (+ T, — AU) medium, followed by incubation in (+ T, + AU) medium for times indicated. This curve shows the loss of acquired immunity. The single point marked B′ corresponds to 90 min in (+ T, — AU) medium followed by 35 min in (— T, + AU) medium, and shows that loss of immunity is independent of the presence of thymine.

Curve C: 140 min in (— T, — AU) medium, followed by incubation in (+ T, + AU) medium for times indicated; shows loss of immunity in the naturally immune cell class.

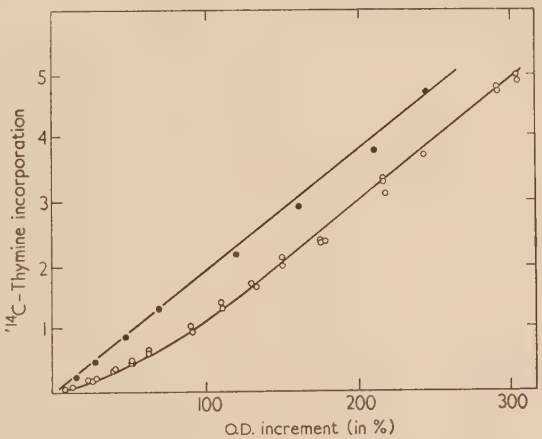

FIG. 6. Incorporation of ^{14}C-thymine added to cultures after different pretreatments.

Lower curve: 90 min in (+ T, — AU) medium, then growth in complete medium containing ^{14}C-thymine (cf. stippled curve in Fig. 7).

Upper curve: 90 min in (+ T, — AU) medium followed by 50 min in (— T, + AU) medium before addition of labeled thymine (cf. dashed curve in Fig. 7). The activities on the ordinate are in arbitrary units, since the lower curve represents two independent experiments.

As before, DNA synthesis was studied by following the incorporation of ^{14}C-thymine. If pre-incubation in the (+ T, — AU) medium is followed immediately by growth in complete medium containing ^{14}C-thymine, DNA synthesis is observed to

increase gradually and to reach its definitive rate only after about one generation time (see Figs. 6 and 7). The net effect of this delay in attaining the full rate of DNA synthesis is to re-establish the normal DNA/mass ratio which had been reduced during pre-incubation (see Fig. 7).

If pre-incubation in the (+ T, — AU) medium is followed by growth in a (— T, + AU) medium before ^{14}C-thymine is added the delay in DNA synthesis is reduced or completely abolished. Thus the definitive rate of thymine incorporation is at once established if the cells are allowed 50 min of growth before labeled thymine is

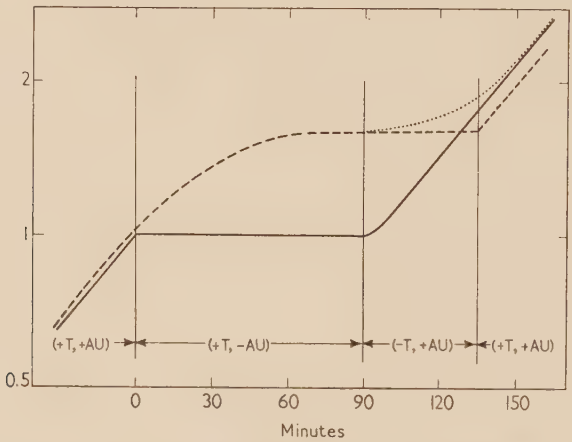

Fig. 7. Schematic representation of DNA synthesis and of the increase in cell mass during an experiment involving three media shifts as indicated on the graph. The solid line shows growth in terms of O.D. increase. The identical curve would obtain if the cells were transferred from the (+ T, — AU) medium *directly* to the complete (+ T, + AU) medium.

The dashed line shows DNA synthesis during the complete experiments. The stippled line illustrates the slow increase in rate of DNA synthesis observed if cells are transferred *directly* from the (+ T, — AU) medium to the complete (+ T, + AU) medium (cf. lower curve in Fig. 6).

added (see Fig. 6). Apparently, the loss of the acquired immunity to thymineless death is paralleled by a gradual increase in the capacity of the culture to synthesize DNA. The significance of these observations at the cellular level will be discussed in the next paper of this issue.

(e) *Cell division in a population initially immune to thymineless death*

We have seen that, in terms of mass increase, the normal growth rate is rapidly established when A and U are added back after incubation for 90 min in the (+ T, — AU) medium. In contrast, cell division is resumed gradually, reaching the normal rate only after 40 to 50 minutes of growth. Thus the division rate increases roughly in the same way as the rate of DNA synthesis does.

The class of cells which survive after prolonged incubation in the (— T, — AU) medium has been investigated separately, since it might be expected to behave more homogeneously than a normal culture. As shown in Fig. 5, about 70% of these cells behave alike in the sense that they regain sensitivity to thymineless death very rapidly. The fact that not all cells behave in this manner could be due to physiological changes occurring during starvation for 140 min in the (— T, — AU) medium. When T, A and U are added after this period the immune cell fraction show signs of division

synchrony: for about 50 min the colony count remained constant and then almost doubled during the next 20 min. However, the second division cycle was not too well defined and after that no sign of synchrony remained.

4. Discussion

The phenomenon of thymineless death which plays such an important role in this study is still poorly understood. One important feature which emerges from the work of several investigators is that loss of viability occurs in the absence of thymine *whether or not* protein and RNA synthesis is allowed to continue. Thus the process leading to death of the cell does not involve *net* DNA synthesis and it seems to be independent of cytoplasmic activities such as the synthesis of new enzymes (as might be imagined to result, e.g. from derepression following removal of thymine). In our present state of ignorance the "killing event" could therefore be described as an irreparable mistake in *attempted* DNA synthesis. Many and careful colony counts have led us to believe that a genuine lag period precedes the phase of strictly exponential loss of viability; i.e. that death is probably not the result of accumulation of *many* independently occurring events. If we assume that complete and correct replication cannot take place in a nucleus in which a single mistake has occurred we can estimate that on an average one such mistake happens every 15 minutes (see Fig. 3). During this period a quantity of DNA containing about 3×10^6 base-pairs would normally replicate. Thus, even in the absence of thymine, this kind of mistake seems to be a relatively unlikely event.

It is known that in *E. coli* cells DNA replication is continuous throughout most of the division cycle (Schaechter *et al.*, 1959; van Tubergen, personal communication, 1960). One would therefore expect that removal of T from a culture of strain $15T^-$, or one of its derivatives, would expose the great majority of the cells to "killing events" of the kind just described. Furthermore, if a small fraction of the population was *not* actively synthesizing DNA, these cells would be expected to resume replication quickly and again became susceptible to thymineless death. The fact that, when T is removed, loss of viability continues exponentially over at least 4 decades is therefore not surprising.

The observation that a fraction of the population is immune to thymineless death in the (— T, — AU) medium suggested that between rounds of replication there might be a significant time interval, *and* that a new round would be initiated only if protein and/or RNA could be synthesized during this interval. The length of the interval might be estimated from the size of the immune fraction; as a first approximation one might equate the immune fraction with the interval expressed as a fraction of the generation time. However, in this way a minimum estimate would be obtained, since residual protein and RNA synthesis, taking place *after* the removal of T, A and U from the culture, might bring cells which at the time of removal were immune into the sensitive class. The fact that between experiments of different design the immune class varies from 3 to 10% shows that the duration of the hypothetical interval between rounds of replication cannot be accurately estimated.

As mentioned in the introduction total DNA increases by at least 40% after inhibition of protein and RNA synthesis. The minimum value of 40% is very close to the theoretical value derived on the assumption that already initiated replication will run to completion but that a new round cannot start in the absence of protein

and RNA synthesis.† Obviously, this agreement between experiment and theory could be fortuitous: for instance, the DNA made after transfer of the cells to the (+ T, — AU) medium could be derived from a precursor pool which happened to be of the right size. However, an analysis of DNA synthesis at the cellular level has shown that such an explanation is unlikely (see the next paper of this issue).

In many experiments the DNA increment exceeds the 40% calculated for a case of complete and instantaneous inhibition of protein and RNA synthesis. Thus in the experiment reported in Fig. 4 the increment was estimated at about 55%. Such an excess may be accounted for by assuming that cells which finish a first round of replication soon after being transferred to the (+ T, — AU) medium enter on a new round before inhibition has become effective. In order to produce a 55 rather than a 40% increase in DNA, the first 20 to 25% of the cells to finish replication would have to begin, and to complete a second round. In this connection it should be noted that 15 to 25% of the cells are observed to divide regularly during incubation in the (+ T, — AU) medium.

The theory on which our discussion has been based may be restated as follows: *protein and/or RNA synthesis is required to initiate but not to sustain DNA replication.* Clearly the strongest evidence in favour of this theory is the finding that complete immunity to thymineless death is gradually built up if DNA synthesis is allowed to proceed in the absence of protein and RNA synthesis (see Figs. 3 and 5). Further support for the theory comes from autoradiographic studies which will be presented in the next paper of this issue.

Now consider the behaviour of a culture when growth is resumed after pre-incubation for 90 min in the (+ T, — AU) medium. If our theory is correct, at the end of the pre-incubation period each DNA unit (genome) should have completed replication, and the cells might be expected to start synthesizing DNA synchronously when A and U are added back to the culture. On this assumption one might expect that the whole cell population would rapidly become sensitive to thymineless death and that, after about one generation of growth, the cells would re-enter the immune state. As we have seen, no such cyclic change in sensitivity is observed; instead the normal sensitivity to thymineless death is re-established gradually in the course of 40 to 50 min of growth (see Fig. 5).

We believe that the slow transition from full immunity to normal sensitivity is a consequence of the equally slow increase in the rate of DNA synthesis (see Figs. 5 and 6). Anticipating the experimental evidence which is contained in the next paper we may simply state that the increase in the rate of DNA synthesis seems to be due to a gradual increase in the *fraction* of the cells which have resumed replication and thereby have become sensitive to thymineless death. As mentioned earlier, during this period of transition the normal DNA/mass ratio is re-established in the culture as a whole. This may well be achieved through the operation of a control mechanism

† This calculation was based on the simplified assumption that DNA replicates at a constant rate throughout the division cycle. Taking one genome as the unit of DNA, and considering a population of unit size growing exponentially with the rate constant k, the amount, Δ, of DNA which remains to be synthesized to complete a round of replication can be expressed as

$$\int_0^{\ln 2} e^{kt}(kt)/\ln 2 \, d(kt);$$

from which we obtain $\Delta = 2 - 1/\ln 2$. Here the value $1/\ln 2 = 1{\cdot}44$ represents the DNA content of the cells at zero time, and the fractional increase in DNA is therefore $2 \ln 2 - 1 = 0{\cdot}39$.

that prevents the *individual* cell from resuming DNA replication until it has re-established the normal DNA/mass ratio.

The characteristic effects on immunity observed when a pre-incubated culture is transferred to a complete medium are also produced if transfer takes place to a (— T, + AU) medium. In fact, the steps which, according to our theory, must precede a new DNA replication cycle, and which bring a cell from the immune to the sensitive state, seem to take place as efficiently in the absence as in the presence of T (see Figs. 5 and 7). Without attempting to define the chemical nature of these steps we can therefore conclude that they are independent of the replication process proper.

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REFERENCES

Anderson, E. S., Armstrong, J. A. & Niven, J. S. F. (1959), In *Virus Growth and Variation*, 9th Symp. Soc. Gen. Microbiol. London.
Barner, H. D. & Cohen, S. S. (1957). *J. Bact.* **74**, 350.
Barner, H. D. & Cohen, S. S. (1958). *Biochim. biophys. Acta*, **30**, 12.
Billen, D. (1959). *Biochim. biophys. Acta*, **34**, 110.
Cohen, S. S. & Barner, H. D. (1954). *Proc. Nat. Acad. Sci., Wash.* **40**, 885.
Goldstein, A., Goldstein, D. B., Brown, J. B. & Chou, Shao-chia (1959). *Biochim. biophys. Acta*, **36**, 163.
Hanawalt, P. C. (1958). Ph.D. thesis, Yale University.
Kanazir, D., Barner, H. D., Flaks, J. G. & Cohen, S. S. (1959). *Biochim. biophys. Acta*, **34**, 341.
Kjeldgaard, N. O., Maaløe, O. & Schaechter, M. (1958). *J. Gen. Microbiol.* **19**, 607.
Schaechter, M., Bentzon, M. W. & Maaløe, O. (1959). *Nature*, **183**, 1207.

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The Normal DNA Replication Cycle. II

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(*Received 14 October 1960*)

Certain predictions made by the theory developed in our first paper have been examined in autoradiographic experiments. First, individual cells were found to continue DNA synthesis for very different periods after protein and RNA synthesis had been inhibited; this agrees with the assumption that, in the absence of protein and RNA synthesis, DNA replication runs to completion and then stops. Secondly, it was found that if a fraction of the cells of a culture is immune to thymineless death a similar fraction is *un*able to synthesize DNA under conditions of inhibited protein and RNA synthesis; this agrees with our assumption that an immune cell is one that has finished a replication cycle and not yet taken the steps necessary to initiate a new one.

It was further predicted that under certain experimental conditions the cells would go through a complete replication cycle when transferred from a thymine deficient to a thymine containing medium. The actual experiment showed that individual cells in fact produced a quantity of DNA which corresponded to a single replication cycle.

When 5-bromouracil was substituted for thymine it was found that the DNA synthesized in the absence of protein and RNA synthesis was hybrid with respect to bromouracil substitution.

1. Introduction

The theory developed in the preceding paper makes definite predictions concerning DNA synthesis in individual cells. To test these predictions two series of autoradiographic experiments were carried out using tritiated thymidine as a specific label for DNA.‖

The first experiment shows how the quantity of DNA synthesized under conditions of inhibited protein and RNA synthesis is distributed in the cell population. As expected, it was found that individual cells contributed very unequally; some would stop synthesizing soon after transfer and contribute little DNA, while other cells continued synthesizing and produced correspondingly more DNA.

The second experiment was designed to analyse the transition from acquired immunity to thymineless death to sensitivity. As shown in the first paper, this transition is paralleled by a slow increase in the rate of DNA synthesis. The present experiment shows that this change in the rate of DNA synthesis is due to a gradual

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‖ The bacterial strains and the abbreviations used to indicate media supplemented in different ways are described on page 147 of the preceding paper.

increase in the *fraction* of cells that actively synthesize DNA; in agreement with our theory, loss of immunity and resumption of DNA synthesis thus seem to go hand in hand. Furthermore, when DNA synthesis is resumed in a (+ T, — AU) medium it can be shown that those cells which participate produce a quantity of DNA corresponding to one complete round of replication and then stop.

In addition to the autoradiographic experiments, preliminary studies of the DNA made in a (+ 5-bromouracil, — AU) medium were made by density gradient centrifugation.

2. Materials and Techniques

The bacterial strains used, the growth media and the bacteriological techniques are described in the preceding paper.

(a) *Autoradiographic procedures*

Tritiated thymidine (360 mc/m-mole) was obtained from the Schwartz Laboratories, Mt. Vernon, N.Y.; in experiments A and B as well as in the control experiment (see p. 158) it was added without carrier to the media to give a final thymidine concentration of 3 μg/ml. The radiochemical purity of this batch of thymidine was not examined at the time it was used, and purification was not attempted. However, the pilot experiment mentioned on page 162 was carried out in Cambridge by Dr. Robert van Tubergen and one of the authors (P.C.H.), and in this experiment a carefully purified thymidine preparation was used. This pilot experiment and our experiment A both showed the *same* large fraction of unlabeled cells. We therefore conclude that the thymidine used in our experiments did not contain radiochemical impurities which under our growth conditions would produce a detectable background of general labeling.

Incorporation of labeled thymidine took place in aerated, 5 ml. cultures. At intervals 0·4 ml. samples were removed and the cells rapidly fixed by squirting the liquid into tubes held at 4°C and containing one drop of 2% formalin.

Standard glass microscope slides (0·8 to 1 mm thick, with one corner filed to designate a "top" side) were soaked for a week in dichromic acid cleaning solution. After thorough rinsing in tap water and then in distilled water the wet slides were dipped in a filtered solution of 0·01% chrome alum + 0·1% gelatin (Knox) at room temperature. The "subbed" slides were allowed to dry in a dust-free room and stored in a dust-free box.

Drops from the formalin-containing tubes were placed on a clean 1% agar surface. After the liquid had been adsorbed by the agar, impression smears were made on "subbed" slides. After drying, the slides were soaked in Carnoy's solution for 20 min at room temperature and then in the following ethanol + distilled water solutions, respectively for 2 min periods: 95% ethanol, 85%, 75%, 50%, 30%, distilled water. The slides were dried overnight at room temperature.

Kodak AR–10 autoradiographic stripping film was placed over the samples by standard procedure in low level lighting from a Wratten 1 safelight (see, e.g. Doniach & Pelc, 1950). Humidity was maintained at 58 to 60% and a 25°C water bath was used for "floating" the emulsion strip. The film was allowed to dry on the slides for three hours in the dark at room temperature in front of a fan. Then the slides were placed in an air- and light-tight box, flushed with dry nitrogen and stored at 4°C.

After an exposure time calculated to give average grain counts of 2 to 4 per labeled cell, the film was developed for 2·5 min at 20°C in Kodak D19b developer and fixed in Kodak non-hardening acid fixer. After rinsing in tap water excess emulsion was carefully trimmed from the edges of the slide and the emulsion allowed to dry. A coverslip was fixed over the emulsion with a drop of 60% glycerol. Cells and grains were easily visible in phase contrast at a magnification × 800. Independent countings of several of the samples by the two authors gave reasonably consistent results.

3. Autoradiographic Experiments

The first series of autoradiograms was prepared by Dr. Schaechter using *E. coli*, strain 15T⁻ taking advantage of the inhibitory effect on protein and RNA synthesis of a transfer from broth to minimal medium (Kjeldgaard, Maaløe & Schaechter, 1958). It is known that mass increase is almost completely blocked for 50 to 60 min after the transfer and that, during this period, DNA increases by about 40%. In experiments with *Salmonella typhimurium* it has further been shown that total RNA drops by 5 to 10% as DNA increases; one may assume that this RNA fraction supplies the cytosine used in DNA synthesis. The experiment of Schaechter consisted in exposing samples of the culture, taken at different times after transfer, to tritiated thymidine for short periods. Autoradiograms were prepared from the samples as described by Schaechter, Bentzon & Maaløe (1959).

These preparations showed that the fraction of cells which incorporated labeled thymidine into trichloroacetic acid (TCA)-insoluble material gradually decreased from 1·0 at time zero to 0·3 to 0·4 at 60 min after transfer. The quantity of thymidine incorporated/min by an actively synthesizing cell decreased only slightly as the fraction of actively synthesizing cells was reduced. From these experiments we conclude that the period during which DNA synthesis continues after transfer varies greatly from cell to cell, that cells that stop synthesizing shortly after transfer contribute little and that cells that continue DNA synthesis for a longer time contribute correspondingly to the overall DNA increase of about 40%. It was not possible from these preparations to obtain reliable measurements for studying the correlation between cell size and the duration of DNA synthesis. Also it should be mentioned that in this experiment total mass increased by about 10% during the first hour after the transfer. Protein and RNA synthesis was thus less efficiently blocked than in previously conducted experiments with *S. typhimurium*. This may account for the fact that even the 60 min sample showed incorporation of thymidine by a considerable fraction of the cells.

The second series of autoradiograms was obtained from rather more complicated experiments: a culture of the triple mutant of *E. coli*, strain 15T⁻A⁻U⁻, growing exponentially in a fully supplemented, (+ T, + AU) medium, was preincubated in (+ T, − AU) medium for 90 min and then transferred to a (− T, + AU) medium. Two portions, A and B, were grown in this medium for 15 and for 45 min respectively before being returned to (+ T, − AU) medium. During the second period in this medium tritiated thymidine (T*) was substituted for thymine, and samples were removed at intervals for autoradiography.

This experiment was designed (1) to test whether the low rate of DNA synthesis observed during the early stages of growth following pre-incubation might be due to participation by a fraction of the cells only; and (2) to determine the quantity of DNA made per cell during the second period in (+ T,* − AU) medium. In order to be able to express this quantity in suitable units of DNA a control experiment was carried out, in which exponentially growing cells were allowed to incorporate tritiated thymidine for various times.

Table 1 presents the data from 6 typical autoradiograms selected from the control series, and from experiments A and B in which the growth periods in (− T, + AU) medium were 15 and 45 min respectively. The most conspicuous feature of these

experiments is that in A less than half of the cells took up any radioactive thymidine, whereas in the control culture and in experiment B nearly all the cells did.

Figure 1 shows the complete results from experiments A and B plotted against the time of incubation in medium containing tritiated thymidine. The values on the ordinate express quantities of newly synthesized DNA estimated from the grain counts as explained below (see section in small print). In the case of experiment A correction was made for the fact that only a fraction of the cells took up any label, and all ordinate values thus refer to average quantities of newly made (labeled) DNA *per actively synthesizing cell*. The unit in which these quantities are expressed was derived from the control experiment and corresponds to one complete round of DNA

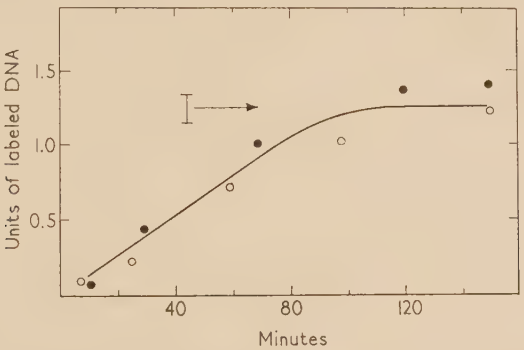

FIG. 1. Experiments A and B are represented by open and filled circles respectively The unit of DNA is defined on page 161. The time axis represents minutes of incorporation in medium containing tritiated thymidine but no A and U. The vertical bar with an arrow shows the interval within which the final yield of labeled DNA is expected to fall (see p. 164).

replication in the presence of the label. As indicated by the curve of Fig. 1, our experiments show that a total of about 1·3 units of DNA was produced per actively synthesizing cell, irrespective of whether all or only a fraction of the cells were engaged in DNA synthesis.

At the end of the 15 and 45 min growth periods in (— T, + AU) medium and after filtration, control samples were incubated for 5 hr in (— T, — AU) medium at 37°C. In experiment A about 70% of the cells and in experiment B 8 to 10% were found to be immune to thymineless death under these conditions (cf. Fig. 5 of our first paper).

Finally, cell division was controlled throughout the experiment by direct colony counts. During the first 90 min in (+ T, — AU) medium the viable cell count increased by about 20%, and during the following 45 min in (— T, + AU) medium a similar small increase was observed; during the second period in (+ T*, — AU) a further increase of about 40% took place. Thus the overall increase was close to one doubling. The significance of these details will be discussed on p. 164.

(a) *Analysis of autoradiograms*

A uniformly labeled population of cells of identical size should produce an autoradiogram in which the number of grains per cell follows a Poisson distribution. In the *control culture* we may assume 100% viability and very nearly the same specific activity of the labeled DNA in all cells. The observed grain distributions were therefore analysed graphically by means of the equation: $\ln (p(x)x!) = x \ln m - m$, in which $p(x)$ is the relative

frequency of the class of cells which produced x grains each and m is the mean value of a Poisson distribution. Using the observed frequencies and plotting the left-hand side of the equation ($P(x)$) against x a straight line should obtain if the $p(x)$ values fit a Poisson distribution. The slope and the intercept of this line should yield two estimates of m. These estimates of course should agree with the arithmetic mean of the grain counts. Data from 6 autoradiograms from the control experiments were analysed and found to satisfy these conditions reasonably well, showing that the observed grain distributions approximate to Poisson distributions (two examples are shown in Table 1 and Fig. 2). With reference to experiments A and B below, it should be particularly noted that in all six distributions the $p(o)$ values agreed with the rest of the $p(x)$ values.

TABLE 1

Autoradiographic data

Grains per cell	Control experiment 1	Control experiment 2	Experiment A 1	Experiment A 2	Experiment B 1	Experiment B 2
0	94	34	400	464	60(17)‡	42(12)‡
1	194	80	84	217†	50	32
2	182	88	51	145	80	32
3	144	75	37	75	93	43
4	83	51	27	59	110	51
5	56	31	11	43	98	82
6	20	10	3	20	69	89
7	8	5	1	10	44	38
8		1		5	11	15
9				1	4	6
Cells scored	781	375	613	1039	619	430
m_a	2·27	2·51	0·79	1·38	3·66	4·23
m_e	2·38	2·48	2·14	2·94	3·78	4·37
m_e/t	0·40	0·50	0·71	0·59	0·76	0·63

m_a = arithmetic mean of grain counts.

m_e = graphically estimated mean; t = time in days of exposure of autoradiographic film.

In control experiments 1 and 2 incorporation of label was continued for 50 and 65 min respectively; the corresponding estimates of m_e/t for total labeling were 0·72 and 0·76 (see p. 161).

In experiment A both samples represent incorporation for 150 min.

In experiment B samples 1 and 2 represent incorporation for 120 and 150 min respectively.

† The probable reason for this excessive value is discussed on page 162.

‡ The figures in brackets are corrected $p(o)$ values assuming that 7% of the cells had remained unlabeled (see p. 162); the effect of this correction is illustrated in Fig. 2.

The graphical method used to estimate the mean values (the m_e of Table 1) permits an evaluation of the error of this estimate. In the control experiment as well as in experiments A and B below, the individual estimates of m are believed to be correct within $\pm$ 10%. A more exact calculation of the standard deviations of the estimates of slopes would be difficult since the observed $p(x)$-values carry extremely different weights.

Considering that cells of an exponentially growing culture contain anywhere from 1 to 2 genomes per nucleus, and since some variation in the number of nuclei per cell was noticed, it was at first surprising that the observed grain distributions did not deviate significantly from Poisson distributions. We therefore calculated the distribution to be expected from the simplifying assumption that replication starts at the time of cell

division and continues at a constant rate throughout the division cycle. Based on this assumption the cell population can be divided into, e.g. 10 classes with respectively 1·0 to 1·1; 1·1 to 1·2; . . . and 1·9 to 2·0 genomes per nucleus. Here the class with least DNA comprises the newly divided cells and is almost twice as large as the class with most DNA (the cells about to divide). In a culture homogeneously labeled with tritiated thymidine each class should give a grain distribution of the Poisson type with a mean value proportional to the average DNA content per cell of the class. Disregarding variation in the number of nuclei per cell, these mean values should vary twofold. Assigning arbitrary mean values from 2·1 to 3·9 to the 10 classes and summing the individual distributions we obtained an approximation to the theoretical grain distribution for the whole population. Analysed graphically as described above this distribution exhibited a slight upward concavity; however, the deviation from the line representing the Poisson distribution with mean 2·9 was small and would completely escape notice in our experiments in which at most 1,000 cells were scored per autoradiogram.

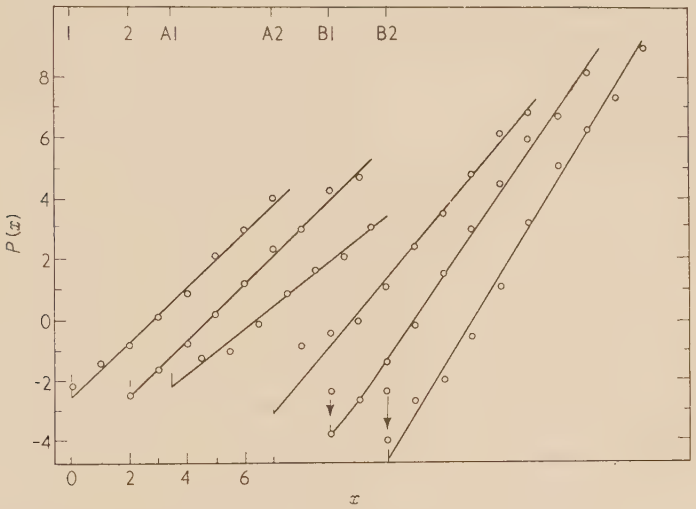

FIG. 2. The six lines represent the grain distributions of Table 1. The x on the abscissa refers to curve No. 1 and indicates the number of grains/cell in the class whose frequency-function, $P(x) = \log_e (p(x)x!)$, figures on the ordinate. The other curves are displaced to the right and on top of the frame the position is indicated of the individual zero-classes. Curves A1 and A2 are based on the assumption that 60% of the cells did not incorporate any label; notice that by this correction fair agreement was obtained between the estimates of $\ln m$ from the slope and of m itself from the intercept. On curves B1 and B2 two $P(o)$ values are shown; the upper ones correspond to the observed $p(o)$, the lower ones to the corrected values shown in brackets in Table 1.

In the control experiment the usual generation time of 42 min was observed, and since the definitive rate of incorporation of thymine into DNA is established without a noticeable lag (see p. 149 of this issue), values for grains per day per *totally* labeled cell could be calculated. The figures 0·72 and 0·76 were obtained from experiments 1 and 2 of Table 1 (50 and 65 min incorporation periods respectively). From the total of 6 autoradiograms, representing incorporation times from 30 to 80 min a weighed mean of 0·753 was obtained. In an exponentially growing culture the *average* DNA content per nucleus is taken to be 1·44 times that of a genome (see p. 159 of this issue). The figure 0·753/1·44 = 0·52 is therefore our estimate of the average number of grains that would be produced per day by a cell which was allowed to incorporate tritiated thymidine throughout one round of replication of its DNA. This figure was chosen as unit on the ordinate of Fig. 1.

Experiment A. In this experiment the zero-fraction is obviously much larger than would correspond to a Poisson distribution. We therefore tested the hypothesis that the cell population studied consisted of a large fraction of cells which had not incorporated any thymidine, plus a fraction of labeled cells giving rise to a grain distribution of the

Poisson type. In the above equation $\ln m$ depends on the *ratio* between the relative frequencies only; thus, plotting $P(x)$ against x, and disregarding the zero-fraction, a straight line should be obtained even if the sum of cells scored included a large fraction of unlabeled cells. The slope of this line would be an estimate of the mean value of the Poisson distribution of the fraction of *labeled* cells. If the unlabeled fraction is large this estimate of the mean will be strikingly high compared with the arithmetic mean.

Experiment A involved the scoring of 12 different autoradiograms, of which two were from an independent pilot experiment (see p. 157). The graphical analysis of the data led to the conclusion that the cell populations could be adequately described as composed of about 60% of unlabeled cells plus a labeled fraction exhibiting a Poisson distribution of grains. Table 1 and Fig. 2 present the scorings of two autoradiograms of this series. A1 with a high and A2 with a very low zero-fraction. The low zero-fraction correlated with an excessively large number of cells associated with single grains, presumably a result of the high background of grains in that preparation. When about 60% of the cells are unlabeled a high background, by adding a "false grain" to, e.g. 10% of *all* cells, increases the one-fraction out of proportion and at the same time markedly reduces the zero-fraction. In Table 1 the postulated heterogeneity with respect to labeling is indicated by the large discrepancy between the arithmetic and the graphically estimated mean values of experiments A1 and A2.

Experiment B. In this case 9 autoradiograms were scored and the data analysed graphically. The grain distributions all closely resembled Poisson distributions, but, generally, the zero-fractions were slightly too large. This was particularly apparent in cases where the estimated mean values were high as in the distributions B1 and B2 presented in Table 1. The best fits to Poisson distributions were obtained by assuming that 5 to 10% of the cells had remained unlabeled and that the rest of the population was homogeneously labeled (see Fig. 2). This high degree of homogeneity in the population is indicated by the close agreement between the arithmetic and the estimated mean values shown in Table 1.

4. Density Gradient Analysis

In a few experiments 5-bromouracil (Bu) was substituted for thymine in media with and without A and U.

First, growth and viability were studied in a (+ Bu, + AU) medium. Optical density (O.D.) was found to increase exponentially at the normal growth rate for about 100 min; after that time the rate slowly decreased. In contrast, the viable count after a small initial increase dropped exponentially with a half-life of about 20 min. At 140 min when the growth rate, in terms of O.D. increment, had been reduced to half only 10% of the cells survived as colony formers. In these respects the process resembles thymineless death in a (−T, + AU) medium. Second, in a (+ Bu, − AU) medium the usual very slight increase in O.D. was observed and *no* loss of viability occurred over a period of 5 hr.

Bromouracil labeled with ^{14}C in the 2-position (Bu*) was used to follow incorporation into the nucleic acids. In the (+ Bu*, + AU) medium the differential labeling (counts/minute/unit increase in O.D.) remained constant over more than a fourfold increase in O.D. This suggests that at least two rounds of DNA replication took place during which Bu replaced T. Consequently, a large fraction of the newly formed DNA should carry the analogue in both strands. In the (+ Bu*, − AU) medium the label was initially incorporated at a rate comparable to that obtained in (+ Bu*, + AU) medium.† However, after 80 to 90 min incorporation had almost stopped. We con-

† Some of our observations indicate that if the medium contains no U, an appreciable part of the label reaches RNA. This finding was not unexpected since it has previously been shown that *E. coli* may convert Bu to U which then may be incorporated into RNA (Wacher, personal communication).

clude that DNA synthesis probably continued in the (+ Bu, — AU) medium in the same manner and to much the same extent as in the (+ T, — AU) medium.

The Bu-substituted DNA synthesized under these conditions was extracted and its density determined by Dr. Cummings. Extraction and centrifugation in the CsCl gradient were carried out as described by Meselson & Stahl (1958).

In the gradient the DNA extracted from cells incubated in a (+ Bu, — AU) medium was found to separate into two *distinct* and *symmetrical* bands. From the positions of the bands the density of the two fractions was calculated to be 1·75 and 1·70, corresponding to half-substituted and to normal DNA respectively. In four samples, one taken just before Bu was substituted for T, and three taken after 15, 40 and 90 min in the (+ Bu, — AU) medium, the DNA recoveries were in good agreement with the expected increase in total DNA. In the 90 min sample the ratio of heavy to total DNA was approximately 0·6; at earlier times the ratio was correspondingly decreased. This preliminary study included control experiments with DNA extracted after prolonged growth in a (+ Bu, + AU) medium. As mentioned above, Bu* was incorporated to an extent indicating that DNA containing Bu in both strands should have been formed; nevertheless, we failed to extract any material banding in the position corresponding to fully Bu-substituted DNA. We have no explanation to offer for this failure.

5. Discussion

The theory presented in our first paper contains the following postulates: (1) that the division cycle of the bacterial cell consists of two periods, one during which a complete round of DNA replication takes place, and one during which *no* DNA is made; and (2) that inhibition of protein and RNA synthesis does *not* interfere with the replication process itself, but prevents the initiation of a new round of replication. Two predictions made by this theory have been examined, both of which concern DNA synthesis in the individual cells of a culture. It was therefore necessary to use autoradiographic techniques.

The first, and also the simplest case is that of a culture in which protein and RNA synthesis is suddenly inhibited. Clearly, the prediction is that cells that, at the time of inhibition, had almost finished replication should continue DNA synthesis for a short time only, whereas cells that had just begun replication should continue synthesis for a longer time and effectively double their DNA under the condition of inhibited protein and RNA synthesis. The experiment of Schaechter confirms this expectation in a qualitative manner, showing that after inhibition of protein and RNA synthesis a gradually decreasing *fraction* of the cells participate in DNA synthesis. As mentioned on page 158 the method used produces temporary inhibition of protein and RNA synthesis only; it is therefore understandable that DNA synthesis does not come to a complete stop at any time during this experiment.

The second prediction is based on our observations on susceptibility and immunity to thymineless death in a (— T, — AU) medium. First it was shown that complete immunity developed gradually in a culture if DNA synthesis was allowed to proceed in the absence of protein and RNA synthesis, i.e. in a (+ T, — AU) medium. This was interpreted to mean that all cells eventually finished their DNA replication, and that they remained in the non-synthesizing state in which they suffered no damage if T was also removed from the medium (see I, p. 153). In other words, susceptibility

to thymineless death was taken to indicate that a cell was actively synthesizing DNA *at the time* when T was removed. Next we observed that the immunity acquired during incubation in (+ T, — AU) medium was lost *gradually* during subsequent growth. If an immune cell is one that has finished a replication cycle and not yet taken the steps necessary to start a new one, *loss* of immunity should indicate that these steps have been taken and that the cell is now able to resume DNA synthesis. Thus, at the time when, e.g. 50% of the cells of a culture have become susceptible the culture should be composed of an equal number of DNA synthesizing and of non-synthesizing cells.

In experiments A and B we observed that about 30% and about 90% of the cells respectively were susceptible to thymineless death at the end of the growth periods in (— T, + AU) medium. These figures then should be compared with the autoradiographic data which showed that about 40% of the cells in experiment A, and 90 to 95% in experiment B actively incorporated tritiated thymidine when transferred to the (+ T*, — AU) medium.

The figures 30 and 40 for the percentages of susceptible and actively synthesizing cells respectively may not be significantly different; nevertheless, it should be pointed out that a likely correction for the effect of cell division reduces the figure 40 considerably. We have seen that about 40% of the cells divided in the (+ T, — AU) medium if the preceding growth period in (— T, + AU) medium was 45 minutes. Presumably, division and resumption of DNA synthesis are correlated events, and we may therefore assume that in experiment A about 40% *of the actively synthesizing cells* divided during the period in which they incorporated the label, and that the rest of the cells did not divide. If this is so, the true percentage of active cells at time zero in experiment A would be 32 and not 40.

The quantity of DNA synthesized during the incorporation of tritiated thymidine may now be compared with that predicted by our theory. The reasoning runs as follows: during the first period in (+ T, — AU) medium replication is thought to run to completion, and each original nucleus should thus be represented by 2 complete genomes (as pointed out on page 153, the few cells that divide during this period probably complete a new round of replication after division). In the second period in the (+ T, — AU) medium a new and complete round of replication is assumed to take place. This should result in the production of 4 half-labeled genomes per original nucleus, and an average of 2 units of DNA per original cell (see p. 159). This figure should be corrected for cell division. Since altogether 60 to 70% of the cells divide during the growth period in (— T, + AU) and the labeling period in (+ T*, — AU) media, the 2 units of DNA per cell reduces to about 1·25. This corrected value agrees reasonably well with the figures 1·2 and 1·4 from experiments A and B respectively (see Fig. 1).

The average curve drawn in Fig. 1 shows that systematically experiment B gave slightly higher values than experiment A. Thus, although the final levels are not significantly different, DNA synthesis probably was somewhat slower in experiment A than in B. This could be due to the average DNA/mass ratio being considerably greater in experiment A than in B; see the schematic diagram (Fig. 6) of the preceding paper.

In general, the results of experiments A and B agree with the predictions of our theory: First, we have shown that if a certain fraction of the cells of a culture is *immune* to thymineless death, a similar fraction is unable to synthesize DNA, and *vice versa*. Second, we find that cells which are believed to begin a new replication

cycle when transferred to a (+ T,* — AU) medium produce a quantity of DNA corresponding to one complete cycle and not more.

Finally, we shall discuss the results obtained by density gradient centrifugation. As explained on page 163, Bu can be substituted for T in a (+ T, — AU) medium without apparently changing the course of DNA synthesis. According to our theory total DNA should increase by about 40% in a (+ Bu, — AU) medium, and the new material should consist of fully Bu-substituted strands paired with "old" strands containing T. In the ideal case of instantaneous and complete inhibition of protein and RNA synthesis no material containing Bu in both strands should be formed. If total DNA increases from 1·0 to 1·4 arbitrary units, we would therefore expect to find 0·6 units of normal DNA and 0·8 units of material containing Bu in one strand only. The ratio of hybrid to total DNA predicted by theory is thus 0·57, a value with which the observed ratio of 0·6 agrees almost perfectly.

This excellent fit should be viewed with some reservation, however: as discussed in our first paper (p. 153), it is likely that a small fraction of the cells finish replication soon after transfer to a (+ T, — AU) medium, then divide and go through a complete new round before stopping. If 20% of the cells went through a second cycle total DNA would increase by about 55% and the expected ratio of hybrid to total DNA would be about 0·67 instead of 0·57. Moreover, a few per cent of the newly synthesized DNA would be fully Bu-substituted. Under our experimental conditions these few per cent probably would not have been detected; nevertheless, in this connection it must be remembered that *no* fully substituted DNA was recovered in the control experiment although such material probably was formed. The best argument against significant quantities of fully substituted DNA being produced during incubation in the (+ Bu, — AU) medium is that the increase in total DNA could be accounted for almost completely by the increase in the hybrid DNA recovered from the individual samples.

The fact that a homogeneous fraction of hybrid DNA was formed during incubation in the (+ Bu, — AU) medium is less ambiguous. It shows that the "excess DNA" produced in the absence of protein and RNA synthesis probably is formed through the normal, semi-conservative replication process first demonstrated by Meselson & Stahl (1958).

The authors wish to thank Dr. Robert van Tubergen for his generous help in carrying out the pilot experiment discussed on page 162. One of us (P.C.H.) acknowledges the award of a U.S. Public Health Postdoctoral Fellowship.

REFERENCES

Doniach, I. & Pelc, S. R. (1950). *Brit. J. Radiol.* **23**, 184.
Kjeldgaard, N. O., Maaløe, O. & Schaechter, M. (1958). *J. Gen. Microbiol.* **19**, 607.
Meselson, M. & Stahl, F. W. (1958). *Proc. Nat. Acad. Sci., Wash.* **44**, 671.
Schaechter, M., Bentzon, M. W. & Maaløe, O. (1959). *Nature*, **183**, 1207.

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Synthesis by *Escherichia coli* of an Abnormal β-Galactosidase in the presence of Thiouracil†

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In the presence of 2-thiouracil, the increase in β-galactosidase activity is inhibited to a greater extent than the corresponding protein assayed immunologically. It seems that the protein synthesized in the presence of the analogue loses some of its enzymic sites. It is suggested that this is a consequence of thiouracil incorporation into RNA templates or eventually into S-RNA.

1. Introduction

Several recent papers indicate that modification of RNA by means of base analogues or nucleolytic enzymes is possible without blocking protein synthesis completely. Chantrenne's (1958) work on penicillinase formation in *Bacillus cereus* suggests that abnormal enzyme can be synthesized in the presence of azaguanine containing RNA. Jeener (1959) has shown that *Bacillus megatherium* bacteriophages grown in the presence of RNase are but slightly infective and contain altered protein constituents (Jeener, private communication).

These agents might leave the RNA molecules relatively unaltered in their chemical function but nevertheless induce major modifications in the genetic information transferred by those molecules from DNA to protein. This would lead to synthesis of proteins of altered structure which could then be directly related to modifications imposed upon the RNA molecules (Matthews, 1957).

In search of a convenient material to test this hypothesis, that certain modifications of RNA molecules degrade directly or indirectly the information content of these molecules and hence lead to the synthesis of degenerated proteins, it was found that 2-thiouracil—a pyrimidine analogue incorporated into RNA (Jeener & Rosseels, 1953; Hamers, 1956), both soluble and microsomal, but not into DNA—inhibits the formation of active β-galactosidase in *Escherichia coli* without affecting total protein synthesis very much. A similar inhibition of β-galactosidase formation by 5-fluorouracil has been reported by Horowitz, Saukhonen & Chargaff (1958). Moreover, Monod (personal communication) had attracted our attention to the fact that the linear growth of *B. megatherium* cultured in the presence of 2-thiouracil (Hamers, 1956) is very reminiscent of linear growth induced by amino acid analogues known to be incorporated in non-functional proteins (Cohen & Munier, 1959).

It was therefore attempted, by combined immunological and enzyme assays, to determine whether or not synthesis of structurally altered inactive enzyme occurs in

† A preliminary note was published elsewhere (Hamers & Hamers-Casterman, 1959).

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the presence of 2-thiouracil (Hamers & Hamers-Casterman, 1959). Immunological and enzymic activity both affect a small region of the protein molecule. Furthermore in *E. coli* β-galactosidase immunological precipitation does not interfere with enzymic activity (Cohn & Torriani, 1952). The two sites are thus distinct in this enzyme, and their eventual alteration, if quantitatively different, must lead to discrepancies between enzyme and immunological assays.

In similar experiments with fluorouracil Gros (1960*a*) and Bussard, Naono, Gros & Monod (1960) have independently shown that this pyrimidine analogue also leads to the synthesis of deficient enzyme molecules.

2. Experimental

The method developed by Cohn & Torriani (1952), for the immunological assay of β-galactosidase proved to be the most convenient and was used throughout this work. In principle it consists in the determination of the amount of enzyme units of a preparation precipitated at the equivalence point by a known volume of antiserum.

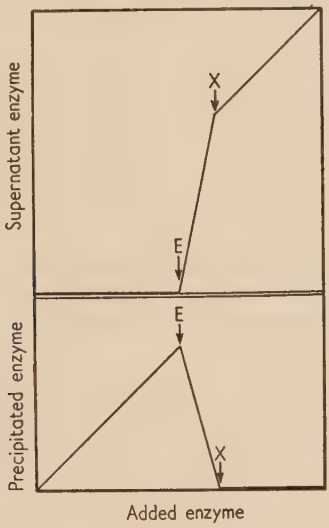

Fig. 1. Schematic determination of the equivalence point E. For all enzyme concentrations below the equivalence point, all the enzyme is precipitated and none appears in the supernatant. For concentrations above the equivalence point, enzyme activity appears in the supernatant and, in the presence of a sufficient excess, no enzyme units at all are precipitated (all points above X).

Increasing amounts of enzyme are added to a series of tubes each containing an identical amount of an appropriate dilution of antiserum. The presence of enzyme units in the supernatant after removal of the immunological precipitate denotes that the equivalence point is exceeded. The graph obtained by plotting the enzyme units in the supernatant against the enzyme units added is schematized in Fig. 1. If for different enzyme preparations the ratio of enzyme sites to immunological sites differs this will be reflected by a displacement of the equivalence point to or from the origin (Fig. 2). This type of immunological assay does not encounter the usual difficulties arising from the use of impure immunization antigen. Moreover, the presence of the serologically cross-reacting protein Pz in *E. coli* extracts is without effect on the determination of the equivalence point since its precipitation occurs only after all β-galactosidase has already precipitated (Cohn & Torriani, 1952).

3. Materials

(a) *Bacteria*

The three strains of *E. coli* used were obtained from Dr. Monod of the Pasteur Institute, Paris.

Strain ML 37 is galactoside permease $^-$, β-galactosidase $^+$, inducible.

Strain ML 30 is galactoside permease $^+$, β-galactosidase $^+$, inducible.

Strain ML 308 is galactoside permease $^+$, β-galactosidase $^+$, constitutive.

The three strains were cultured at 34°C on a mineral medium (Monod, Cohen-Bazire & Cohn, 1951) to which 0·4% maltose is added as carbon source. Under our experimental conditions, turbidity remains a valid estimate of protein synthesis since there is no preferential inhibition of protein synthesis. Synthesis of β-galactosidase was promoted by the non-metabolized inducer *iso*propylthiogalactoside (IPTG) in the ML 37 strain and by lactose in the ML 30 strain.

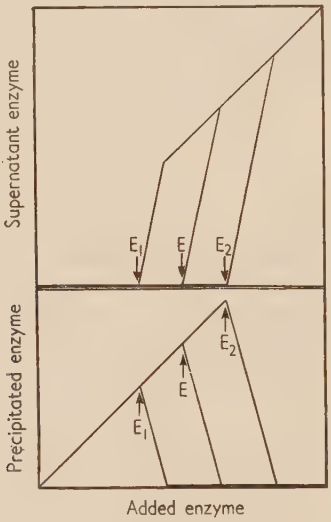

FIG. 2. Theoretical displacement of the equivalence point to (E_1), or from (E_2), the origin for the enzyme preparation having a lower or a higher enzyme site/immunological site ratio than normal enzyme.

(b) *Preparation of cell extracts*

100 ml. bacteria in exponential growth phase (1 to 2×10^9 cells/ml.) or after growth on thiouracil 0·005 M are harvested by sedimentation, washed twice with cold medium and concentrated tenfold in 0·1 M-phosphate buffer to which 0·001 M-thioglycolic acid and 0·001 M-$MnCl_2$ and $MgCl_2$ are added. The Mn^{2+} (Rickenberg, 1959) and Mg^{2+} ions protect β-galactosidase from denaturation at low protein concentrations whereas thioglycolic acid apparently prevents polymerization of the enzyme molecules (Hu, Wolfe & Reithel, 1959). The bacteria were disrupted by 5 min treatment in a Raytheon 10 kc magnetostrictive oscillator. The extract was centrifuged for 20 min at 10,000 rev/min at 0°C and the pellet discarded. These extracts were stable at 4°C and were assayed for β-galactosidase by the enzyme and immunological methods described.

(c) *Analytical methods*

(i) *Enzyme assay*

Routine enzyme assays were performed with *o*-nitrophenylgalactoside (Lederberg 1950) as substrate. The substrate was used at a final concentration of M/475 in 0·05 M-sodium phosphate buffer pH 7·0. Hydrolysis was followed by the increase in O.D. at

420 mμ. The unit of activity is defined as the amount of enzyme that will hydrolyse 10^{-9} moles of substrate/min at 28°C. β-galactosidase formation in bacteria was measured in toluene-treated fractions (Novick & Wiener, 1957).

(ii) *Immunological assays*

Enzyme for immunization purposes was obtained by purification of the crude extract (ML 30) by repeated $(NH_4)_2SO_4$ fractionation (s = 0·28 to 0·35). Addition of ^{35}S labelled extract of uninduced bacteria allowed purification to be followed. This was stopped after removal of 80% of the radioactivity. Immune serum was prepared by the method of Freund in which the *mycobacteria* were omitted from the adjuvant. Pre-immunization serum was used in all control experiments. Assay of the immunological β-galactosidase was made by the following modification of the method of Cohn & Torriani (1952).

Centrifugation of the immunological precipitates is facilitated by the use of a carrier precipitate (Monod, private communication). 0·4 ml. of the following mixture is added to each of a series of heavy pyrex test tubes (capacity 1·2 ml.),

antiserum or pre-immunization serum	1 vol
phosphate buffer	2 vol
tobacco mosaic virus (TMV) 1% } carrier	4 vol
anti TMV serum } carrier	1 vol

This mixture is always made up immediately before use. The type of carrier precipitate is of little importance and the TMV and anti-TMV carrier can be conveniently replaced by others such as serum albumin and anti-serum albumin which is currently used in Dr Monod's laboratory at the Pasteur Institute where Cohn developed his method.

Each of the test tubes is finally completed to 1 ml. with variable amounts of buffer and enzyme extract. The tubes are incubated at 37°C for 2 hr and left at 4°C overnight. Centrifugation at 10,000 rev/min for 20 min eliminates the immunological precipitate. The non-precipated enzyme is assayed on the supernatant. The control tubes, where the antiserum is replaced by pre-immunization serum, indicate the initial amounts of enzyme per tube. A typical experiment is shown in Table 1.

Table 1

Tube number	1	2	3	4	5	6	7	8	9	10
Antiserum, ml.	0·1	0·1	0·1	0·1	—	0·1	0·1	0·1	0·1	—
Normal serum, ml.	—	—	—	—	0·1	—	—	—	—	0·1
Buffer	0·8	0·77	0·74	0·7	0·7	0·65	0·6	0·5	0·4	0·4
Added enzyme, ml.	0·1	0·13	0·16	0·2	0·2	0·25	0·3	0·4	0·5	0·5
Added enzyme units	94·4	123	153	189	189	236	284	378	472	466
Supernatant enzyme	0	0	0	18	196	192	270	384	455	466

4. Results

Both induced and constitutive β-galactosidase synthesis are extensively inhibited by 2-thiouracil but their inhibition can be completely reversed by uracil (Fig. 3).

Bacterial extracts were made as described under Methods. After growth in the presence or absence of the analogue the bacteria were harvested and the number of enzyme units precipitable by 1 ml. of antiserum was determined. In inducible strains the inducer is added at the same time as the thiouracil. Figure 4 shows that in the extracts derived from bacteria grown with 2-thiouracil the amount of enzyme precipitated at the equivalence point by a known amount of antiserum is decreased.

The same result was obtained in experiments in which the control extracts were replaced by extracts of bacteria grown on equimolar amounts of thiouracil and uracil (Table 2). To obviate the objection that these results are due to the fact that in all

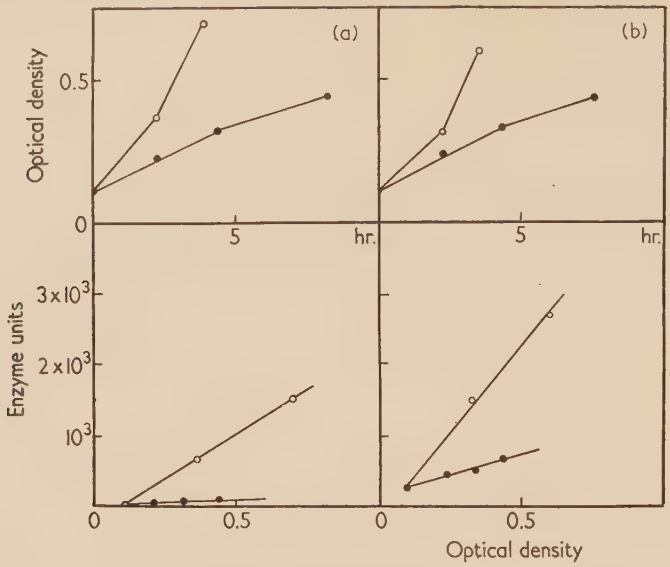

FIG. 3. Growth (above) and differential rate of enzyme synthesis (enzyme units versus growth) in absence —○— or presence —●— of thiouracil. (a) ML 37 inducible strain. (b) ML 308 constitutive strain.

TABLE 2

Strain	Culture conditions	Extract diluted with	Units β-galactosidase precipitated by 1 ml. antiserum at equivalence point (10^5)
ML 308	control	buffer	1·27 1·30
ML 37 induced	control	buffer	1·30
		uninduced ML 37 extract	1·32
	2-thiouracil 0·005 M + uracil 0·005 M	buffer	1·37
ML 308	2-thiouracil 0·005 M	buffer	0·99 1·04
ML 37 induced	2-thiouracil 0·005 M	buffer	0·73 0·88 0·80

experiments the control extracts containing considerable amounts of active enzyme have to be diluted ten- to twenty-fold, whereas the extract from induced thiouracil grown cells does not have to be diluted at all, other experiments were run in which

the control extract was diluted with an extract from uninduced bacteria. Again the results obtained are the same (Table 2).

The β-galactosidase of the thiouracil-grown bacteria thus contains less enzymic activity per immunological site than the enzyme of the control preparation. This fact alone implies, if *in vivo* or *in vitro* inactivation or degradation of the enzyme does not occur, that a certain number of enzyme sites are lacking or are less active in the protein from thiouracil grown cells. Several arguments tend to exclude such inactivation.

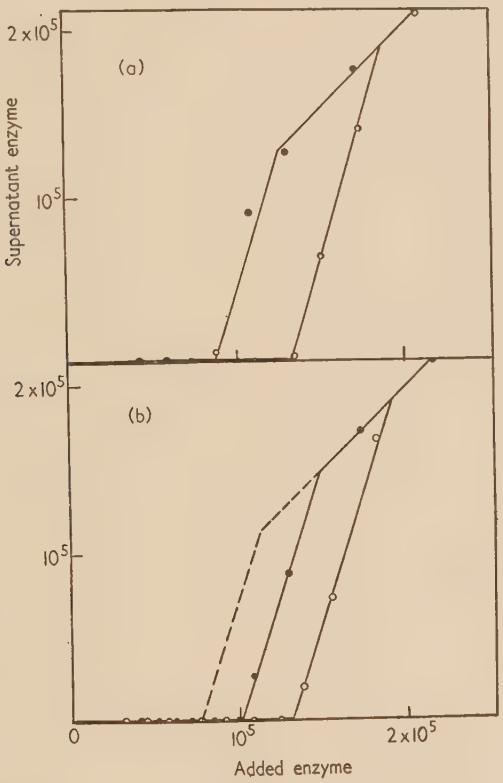

FIG. 4. (a) Displacement of equivalence point for thiouracil-grown enzyme —●— versus control enzyme —○—, ML 37 inducible strain. (b) Displacement of equivalence point for thiouracil-grown enzyme —●— versus control enzyme —○—, ML 308 constitutive strain. Computed displacement of equivalence point of thiouracil-grown enzyme after deduction of 45% for normal enzyme existing before the addition of the analogue is shown by — — — .

(a) In vitro

(i) During the extraction process there is no loss of enzyme activity ; on the contrary the extract always contains slightly more (approximately 10%) onzyme units than could be assayed on the intact cells.

(ii) Upon storage at 4°C the extracts lose very little activity (less than 5% per day).

(iii) The amount of enzyme units precipitated by a known volume of antiserum is very reproducible from one experiment to the other.

(iv) Thiouracil itself has no detectable effect on β-galactosidase activity.

(b) In vivo

(i) Denaturation would lead to a more severe effect of 2-thiouracil on constitutive β-galactosidase, of which a considerable proportion pre-exists at the moment of addition of the analogue, than on the inducible enzyme.

(ii) If the amount of constitutive β-galactosidase present at the time of addition of the analogue is deduced from the total amount of enzyme, it will be noted that the effect of 2-thiouracil on equivalence point displacement is identical for the constitutive and inducible strains (Fig. 4).

One may thus reasonably conclude that the enzyme synthesized in the presence of thiouracil is abnormal and contains fewer or less active sites. A minor modification of this hypothesis is that certain active sites are very labile and are destroyed before they can be assayed. We have not been able to detect quantitative modifications of the Michaelis constant of the abnormal enzyme nor of its affinity for different substrates (Fig. 5). This would suggest that in thiouracil-grown enzyme, either some of

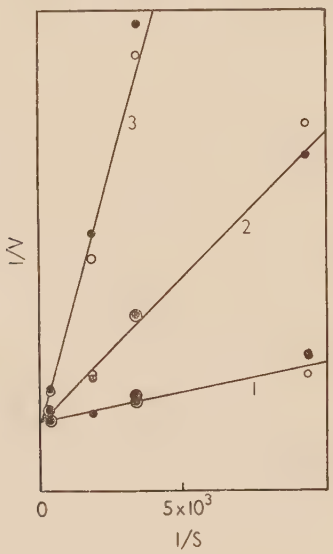

Fig. 5. Lineweaver-Burke plot (Lineweaver & Burke, 1939) of *o*-nitrophenylgalactoside hydrolysis by control —○— and thiouracil-grown —●— enzyme,

(1) in absence of competitive inhibitor,
(2) in presence of 0·04 M-lactose,
(3) in presence of 0·001 M-*iso*propylthiogalactoside.

the active sites have completely lost their affinity for the substrates, while others have remained intact keeping both affinity and function, or that affinity and function have been affected in the same ratio. We do not know how these abnormal sites are distributed among the protein molecules. It is interesting to note, however, that no active enzyme molecules deprived of immunological properties are detected in extracts from thiouracil-grown cells.

Another type of abnormal enzyme which we have no means of detecting might be present in the extracts, *viz.* having lost both immunological and enzyme sites. This would offer a satisfactory explanation to the puzzling fact that total detectable (immunological) enzyme synthesis remains very low in the presence of thiouracil.

Nevertheless, another hypothesis, namely that enzyme repression or induction are mediated by some specific RNA (De Deken-Grenson, private communication; Pardee & Prestidge, 1959) and are therefore sensitive to base analogues (Gros, 1960*b*), must not be neglected. This is also hinted by the fact that thiouracil has a greater inhibitory effect on inducible β-galactosidase synthesis than on constitutive synthesis (Figs. 3 and 4).

5. Discussion

As mentioned previously, the most appealing hypothesis to explain the effect of thiouracil on protein structure is that its incorporation into RNA molecules modifies the information transferred from the template molecules to the proteins. We shall consider certain aspects of this hypothesis which is a probable one since Naono & Gros (1960) and Bussard *et al.* (1960) have independently shown that a quite different pyrimidine analogue, 5-fluorouracil, has a similar effect on not only β-galactosidase but also alkaline phosphatase synthesis.

It is likely that the replacement of uracil by thiouracil (1 to 2% in *E. coli*) in a number of locations in RNA provokes a proportionate number of errors when the genetic information is passed from the 4 nucleotide form of the nucleic acid to the 20 amino acid form of the protein. From what we know from recent studies on mutant proteins (Hunt & Ingram, 1958; Tsugita & Fraenkel-Conrat, 1960) it is likely that the errors reside in amino acid sequence rather than in secondary or tertiary structure. These mistakes might be called translation errors to distinguish them from the different replication errors which happen when genetic information is handed down from nucleic acid to nucleic acid.

When thiouracil is incorporated into TMV or influenza RNA, there is but little decrease in the infective titre of the virus produced (Jeener, 1959; Amos & Vollmayer, 1958). It is equally striking to find that even extensive incorporation of 5-bromo- and 5-iodouracil (Litman & Pardee, 1956; Luzzati, 1957) in bacteriophage and bacteria leads to relatively few mutations. On the contrary nitrous acid, which deaminates amino-bases into keto-bases, is a very efficient agent for the production of mutations in TMV (Gierer & Mundry, 1958) and bacteriophage (Vielmetter & Schuster, 1960). These replication errors (mutations) must arise as a consequence of base mispairing during multiplication of polynucleotide molecules (Freese, 1959; Lavallee, 1960).

Although the pathway by which the genetic information is transferred from nucleic acid to protein is still obscure, it is evident that translation errors as opposed to replication errors can even happen *en route* at a remote distance from the template molecule. A typical error has been described by Sharon & Lipmann (1957) in which the activation enzyme confuses tryptophan and its analogue azatryptophan and promotes the incorporation of the latter into protein. Error in protein synthesis due to incorporation of base analogues can conceivably arise either directly at the level of polynucleotide template molecules or the level of eventual adaptor molecules. Indeed, if alignment of amino acids upon a template necessitates specific intermediate polynucleotide adaptor molecules (Crick, 1958), each of these molecules must possess a region which is specific for one amino acid or its "activated" form. Alteration of this specific region might lead to conversion of one specificity to another with, as a necessary consequence, a translation error consisting in the replacement of one amino acid by another in protein molecules.

At present our knowledge of the mechanism of protein synthesis is too incomplete and it seems irrevelant to discuss in further detail whether thiouracil alters information in template molecules or whether, on the contrary, it affects protein structure by acting on specific sites of small polynucleotide molecules such as S-RNA or Crick's hypothetical adaptors.

We wish to thank Dr. Monod for a gift of IPTG and for the bacterial strains used; and Drs. Jeener and Chantrenne for many helpful discussions.

REFERENCES

Amos, H. & Vollmayer, E. (1958). *Virology*, **6**, 337.
Bussard, A., Naono, S., Gros, F. & Monod, J. (1960). *C.R. Acad. Sci., Paris*, **250**, 4049.
Chantrenne, H. (1958). *Biochem. Pharm.* **1**, 233.
Cohen, G. N. & Munier, R. L. (1959). *Biochim. biophys. Acta*, **31**, 347.
Cohn, M. & Torriani, A. M. (1952). *J. Immunol.* **69**, 471.
Crick, F. H. C. (1958). *Symp. Soc. Exp. Biol.* **12**, 138.
Freese, E. (1959). *J. Mol. Biol.* **1**, 69.
Gierer, A. & Mundry, K. W. (1958). *Nature*, **182**, 1457.
Gros, F. (1960*a*). In *Dynamik des Eiweisses*, Colloquium der Gesellschaft für physiologische Chemie, **10**, 82. Berlin: Springer-verlag.
Gros, F. (1960*b*). In *Dynamik des Eiweisses*, Colloquium der Gesellschaft für physiologische Chemie, **10**, Berlin: Springerverlag.
Hamers, R. (1956). *Biochim. biophys. Acta*, **21**, 170.
Hamers, R. & Hamers-Casterman, C. (1959). *Biochim. biophys. Acta*, **33**, 269.
Horowitz, J., Saukhonen, J. J. & Chargaff, E. (1958). *Biochim. biophys. Acta*, **29**, 222.
Hu, A. S. L., Wolfe, R. G. & Reithel, F. J. (1959). *Arch. Biochem. Biophys.* **81**, 500.
Hunt, J. A. & Ingram, V. M. (1958). *Nature*, **181**, 1062.
Jeener, R. (1959). *Biochim. biophys. Acta*, **32**, 106.
Jeener, R. & Rosseels, J. (1953). *Biochim. biophys. Acta*, **11**, 438.
Lavallee, R. (1960). *C.R. Acad. Sci., Paris*, **250**, 1134.
Lederberg, J. (1950). *J. Bact.* **60**, 381.
Lineweaver, H. & Burke, D. (1939). *J. Amer. Chem. Soc.* **56**, 658.
Litman, R. & Pardee, A. (1956). *Nature*, **178**, 529.
Luzzati, D. (1957). *C.R. Acad. Sci., Paris*, **245**, 1466.
Matthews, R. E. F. (1957). *Biology and Chemistry of Purines*. London: Churchill.
Monod, J., Cohen-Bazire, G. & Cohn, M. (1951). *Biochim. biophys. Acta*, **7**, 585.
Naono, S. & Gros, F. (1960). *C.R. Acad. Sci., Paris*, **250**, 3889.
Novick, A. & Weiner, M. (1957). *Proc. Nat. Acad. Sci., Wash.* **43**, 553.
Pardee, A. B. & Prestidge, L. S. (1959). *Biochim. biophys. Acta*, **36**, 595.
Rickenberg, H. V. (1959). *Biochim. biophys. Acta*, **35**, 122.
Sharon, N. & Lipmann, F. (1957). *Arch. Biochem. Biophys.* **69**, 219.
Tsugita, A. & Fraenkel-Conrat, H. (1960). *Proc. Nat. Acad. Sci., Wash.* **46**, 636.
Vielmetter, W. & Schuster, H. (1960). *Z. Naturf.* **15b**, 304.

J. Mol. Biol. (1961) **3**, 175–184

Two-stranded Helical Configurations for Ribonucleic Acid

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X-ray diffraction patterns from *E. coli* soluble RNA are similar to the disoriented patterns from DNA in the *B* configuration. A number of soluble RNAs have been shown to contain an approximately equal number of moles of adenine and uracil and of guanine and cytosine. Except for the replacement of thymine by uracil these ratios are the same as those observed for DNA. Because of these similarities in chemical composition and X-ray diffraction pattern, and those in the chemical structure of RNA and DNA, an attempt was made to build a two-stranded helical model for RNA that was as similar as possible to the DNA *B* configuration.

In order to accommodate the extra hydroxyl of the RNA it was necessary to make a number of small modifications to the DNA model. The base-pairs were moved by 0·2 Å away from the helix axis and their tilt and twist altered by a few degrees. The pucker of the sugar ring was changed and the distance of the phosphate group from the helix axis increased. The axial translation and rotation per nucleotide pair were increased by 0·1 Å and 1°. The cylindrically averaged squared Fourier transform of the RNA model was very similar to that of the DNA *B* model. Although there is no stereochemical reason why RNA cannot adopt a molecular configuration similar to the *A* configuration of DNA, there is no X-ray evidence to suggest that it does.

1. Introduction

X-ray diffraction patterns have been obtained from DNA from a wide variety of sources (Hamilton *et al.*, 1959); two types of pattern have been observed and two distinct though related configurations derived from them and called *A* and *B*. These configurations are based on specific pairing between complementary chains of bases (Watson & Crick, 1953). X-ray diffraction patterns from *E. coli* soluble RNA (S-RNA) show little orientation (Brown & Zubay, 1960). The positions of the rings in these patterns and their relative intensities are similar to those obtained from DNA specimens in which the molecules are in the *B* configuration and in random orientation. Various determinations of the nucleotide composition of S-RNAs from yeast, *E. coli* and rat liver are summarised in Table 1. For any particular organism they differ by more than the expected errors in the nucleotide estimation indicating differences in the material on which the determinations were made. This could be due to different amounts of contamination by high molecular weight RNA and to differences in the relative concentrations of the various fractions which have been shown to exist in low molecular weight RNA (Lipshitz & Chargaff, 1960). For two-thirds of the determinations in Table 1 the ratios of adenine to uracil and of guanine to cytosine are both within 10% of unity. Therefore except for the replacement of thymine by

uracil these base ratios are similar to those observed for DNA (Chargaff, 1955). The covalent bonding is similar in RNA and DNA (Brown & Todd, 1955). These similarities in X-ray diffraction pattern and in chemical composition and structure make it of interest to know if it is stereochemically possible for RNA to adopt a two-stranded helical configuration similar to the *B* configuration of DNA.

Table 1

Nucleotide composition of S-RNAs from yeast, E. coli *and rat liver*

S-RNA Source	A	U	G	C	pU	$\frac{A}{U}$	$\frac{G}{C}$	$\frac{A+U}{G+C}$	Reference
Yeast	1·00	1·01	1·25	1·19	0·14	0·99	1·05	0·82	Osawa (1960*a*)
Yeast	1·00	1·00	1·12	1·04	0·10	1·00	1·08	0·93	Davis, Carlucci & Roubein (1959)
Yeast	1·00	1·08	1·58	1·53	0·21	0·93	1·03	0·67	Monier, Stephenson & Zamecnik (1960)
Yeast	1·00	1·12	1·52	1·17	0·27	0·89	1·30	0·79	Osawa (1960*b*)
E. coli	1·00	0·96	1·55	1·43	0·06	1·04	1·08	0·66	Osawa (1960*a*)
E. coli	1·00	0·74	1·58	1·42	0·10	1·35	1·11	0·57	Dunn, Smith & Spahr (1960)
Rat liver	1·00	0·92	1·48	1·46	0·20	1·09	1·01	0·65	Osawa (1960*a*)
Rat liver	1·0	1·0	1·8	1·7	0·2	1·0	1·1	0·6	Singer & Cantoni (1960)
Rat liver	1·00	0·81	1·45	1·35	0·15	1·24	1·07	0·65	Lipshitz & Chargaff (1960)†

A, U, G, C and pU are the number of moles of adenylic acid, uridylic acid, guanylic acid, cytidylic acid and pseudouridylic acid per mole of adenylic acid.

† Mean of values for 4 fractions.

It is unlikely that RNA can exist in exactly the same molecular configuration as the *B* form of DNA described by Langridge *et al.* (1960) because there is not enough room for the extra hydroxyl attached to C_2 of the ribose sugar. More precisely the oxygen atom (O_6 in Fig. 1) of this hydroxyl group would be about 2·5 Å from the base atom C_4' (Fig. 1) of the preceding nucleotide if this contained a pyrimidine (for a purine the atom is C_8' and the distance about 2·6 Å). This is about 0·5 Å less than the sum of the van der Waals radii of the oxygen and carbon atoms. In this work an attempt has been made to accommodate the hydroxyl group without making any major changes in the DNA *B* configuration.

2. Stereochemical Assumptions

The configurations of the base-pairs and the deoxyribose part of the sugar were assumed to be as derived by Spencer (1959*a* and *b*); it was assumed that apart from the replacement of the 5-methyl group by a hydrogen atom the stereochemistry of thymine and uracil would be identical. It may be that the small amount of 5-ribosyl-uracil (pseudouridine) which has been found in S-RNA has a quite different structural role from the normal 3-ribosyl-uracil. However this need not be so (Spencer, private communication), since a base-pair formed by adenosine and 5-ribosyl-uracil can be made exactly equivalent to the Watson & Crick pairs if it is assumed that the H-bonding is between $(NH_2)_6$ (adenine) and O_2 (uracil) and between N_1 (adenine) and N_1 (uracil). In Fig. 1 this is equivalent to rotating the pyrimidine though 180° about the line joining C_4 and N_1. In the sugar the hydroxyl at C_2 was assumed to bear a cis-relation to the oxygen at C_3 (Brown & Todd, 1955). The bonds and angles in the

phosphate group were based on the structure determinations of dibenzyl phosphoric acid (Dunitz & Rollett, 1956), sodium triphosphate II (Davies & Corbridge, 1958) and sodium triphosphate I (Corbridge, 1960). The sugar-phosphate chain was assumed to be made up of 3′5′ phosphodiester bonds (Brown & Todd, 1955). Models of scale 4 cm to 1 Å were used as described by Langridge *et al.* (1960).

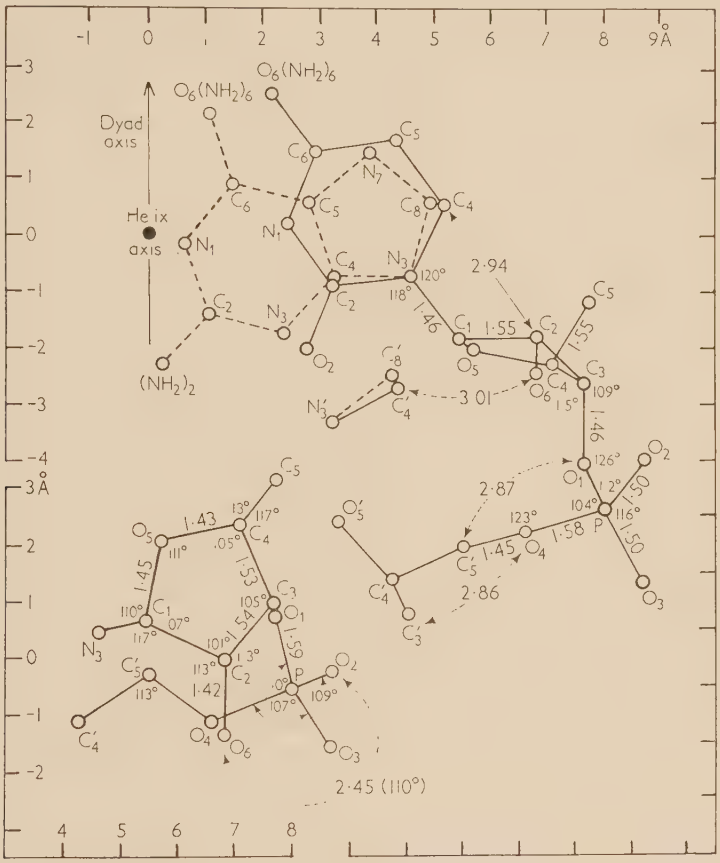

Fig. 1. Projections of a nucleotide of the RNA model on planes perpendicular to the helix axis and to the dyad axis. Bond lengths and angles and short non-bonded interatomic distances are shown.

3. Details of the Final RNA Model

A configuration found for RNA was closely similar to the DNA *B* configuration. Projections of the model with interatomic distances and angles are shown in Fig. 1 and the co-ordinates of the repeating unit are given in Table 2. The cylindrically averaged squared Fourier transform was calculated from these co-ordinates using the atomic scattering factors (Langridge *et al.*, 1960) for atoms immersed in water. It differs only in detail from the DNA transform which is plotted with it in Fig. 3. The method of derivation and the stereochemistry of the final model are discussed in the following sections.

4. The Position of the Bases

In a right-handed orthogonal set of axes XYZ with origin O, OZ is taken as the helix axis of the RNA and OX as the dyad axis of one of the nucleotide pairs. The distance of the base-pair in this nucleotide-pair from the helix axis is defined as OB, the distance of N_3N_9 from O (Fig. 3).

TABLE 2

Cylindrical polar coordinates (R, ϕ, Z) of the atoms in the RNA nucleotide

Group	Atom	R(Å)	ϕ(°)	Z(Å)
Phosphate	P	9·40	59·3	− 0·68
	O_1	8·70	62·5	0·66
	O_2	9·60	66·0	− 1·67
	O_3	10·69	55·4	− 0·35
	O_4	8·47	52·0	− 1·27
Sugar	C_1	5·78	71·5	0·53
	C_2	7·09	75·4	− 0·17
	C_3	8·13	71·6	0·84
	C_4	7·46	72·5	2·21
	C_5	7·84	82·0	3·01
	O_5	6·07	70·6	1·95
	O_6	7·26	70·8	− 1·46
Purine	O_6, $(NH_2)_6$	2·32	153·8	− 0·20
	C_6	1·69	121·2	− 0·04
	C_5	2·88	101·9	0·08
	N_7	4·15	109·5	0·08
	C_8	4·96	97·0	0·23
	N_9	4·64	81·6	0·34
	C_4	3·30	77·6	0·25
	N_3	2·91	54·5	0·29
	C_2	1·76	38·7	0·17
	N_1	0·64	75·5	0·01
Guanine	$(NH_2)_2$	2·43	6·09	0·22
Pyrimidine	O_6, $(NH_2)_6$	3·14	139·0	− 0·156
	C_6	3·20	115·9	0·00
	N_1	2·40	93·6	0·10
	C_2	3·36	74·6	0·27
	O_2	3·45	53·8	0·36
	N_3	4·64	81·6	0·34
	C_4	5·14	96·0	0·25
	C_5	4·55	110·9	0·08

The helix is right-handed with a translation and rotation per residue of 3·44 Å and 37°. The coordinates of the dyad axis are (R, o, o).

In the DNA model the base-pair is tilted slightly about OX. Positive tilt is defined as the sense of a right-handed screw along OX. The bases in each base-pair are twisted about the hydrogen bonds. The twist axis always intersects OX at right angles and its position is defined by OA (Fig. 3). Positive twist is defined as the sense of a right-

handed screw along AC (Fig. 3). The effect of positive twist on an untilted base-pair is shown in Fig. 3; areas of the base-pair above and below the plane of the paper are indicated + and — respectively. The position of a second base-pair is defined by two further parameters: the axial translation and rotation per nucleotide pair.

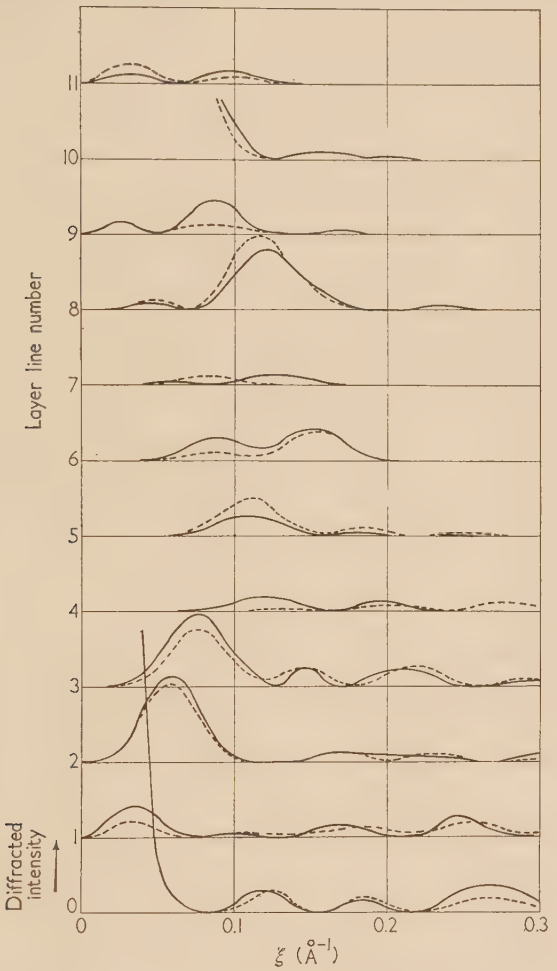

FIG. 2. Comparison of cylindrically averaged squared Fourier transforms of RNA (full-line) and DNA *B* (dashed line, where different from RNA) models.

In Table 3 the values of these parameters are given for both the DNA *B* configuration and the RNA model projected in Fig. 1. All these modifications of the DNA base parameters were chosen because they caused the distance O_6 — C_4' to increase. The increases in OB and the axial translation and rotation per residue increase the distance between the C_1 atoms of successive sugars. Therefore the size of these changes is limited by the length of the fully extended sugar-phosphate chain. The degree of twist and tilt were chosen so that there were no distances between atoms in successive base-pairs less than the normal van der Waals values. The distortions of the hydrogen bond lengths and angles from the values given by Spencer (1959*b*) are as follows:

(a) the distance between the hydrogen donor and acceptor atoms—less than 0·08 Å,
(b) the angle subtended at the donor atom by the acceptor atom and an atom (other than hydrogen) covalently bonded to the donor atom—less than 4°,

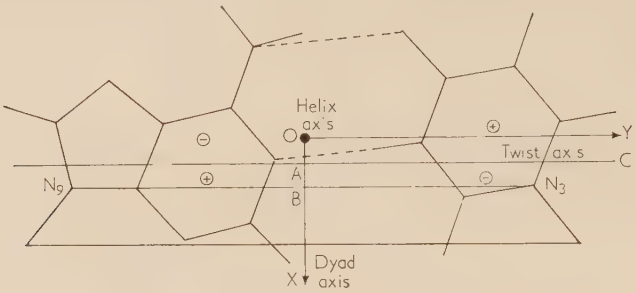

Fig. 3. Diagram to illustrate the position, twist and tilt of the base-pairs (see Section 4).

(c) the angle subtended at the donor atom by the acceptor atom and the hydrogen atom involved in the hydrogen bond—less than 9°.

The distortion in (c) was calculated on the assumption that base hydrogen atoms remain in the plane of the base. If the amino groups can rotate about the bonds joining these groups to the base the maximum distortion given in (c) can be reduced to about 5°; Zubay (1958*a* and *b*) considered this problem in a similar context, and it appears that little precise information is available. The inter-base hydrogen bond lengths and angles are given in Table 4.

Table 3

Comparison of base-pair parameters (defined in Section 4) in the RNA and DNA B configurations

	DNA *B*	RNA
Tilt	+ 2°	+ 4°
Twist	− 5°	− 6°
OB (Fig. 2)	+ 0·6 Å	+ 0·7 Å
OA (Fig. 2)	0·0 Å	0·5 Å
Axial translation per nucleotide	3·36 Å	3·44 Å
Axial rotation per nucleotide	36°	37°

The RNA configuration is non-integral with 9·73 residues per helix pitch.

5. The Configuration of the Sugar-Phosphate Chain

From its orientation in DNA the sugar ring has been rotated through a few degrees about the glycosidic link in an anti-clockwise direction as viewed from N_3 to C_1. This change increases O_6—C_4' but for a given configuration of the sugar ring it also increases the distance between C_3 and C_5' of successive sugars (Fig. 1). This rotation also decreases the distance between C_2 (sugar) and C_4 (pyrimidine of the same nucleotide)

by 0·10 Å from its value in DNA to 2·94 Å. It is unlikely that further reduction of this distance is possible.

It has been shown (Spencer, 1959*a*) that puckering of the sugar ring with either C_2 or C_3 about 0·5 Å out of the plane containing the other four atoms is stereochemically favoured. In the RNA model the pucker differs from that in DNA; C_3 is out of plane on the opposite side of the sugar ring from C_5. This is the pucker which makes the distance C_5'—C_3 a minimum for a given orientation of the sugar ring about the glycosidic link. The other three possible puckers all result in C_5'—C_3 being increased

TABLE 4

Inter-base hydrogen bond lengths and angles

Bond lengths (Å)			Bond angles (°)	
Donor	Acceptor			
N_6(A)	O_6(U)	3·08 (3·06)	$C_6N_6 \ldots O_6$	113 (115)
			$HN_6 \ldots O_6$	14 (8)
N_1(U)	N_1(A)	2·94 (2·94)	C_6N_1(U) $\ldots N_1$	112 (115)
			C_2N_1(U) $\ldots N_1$	123 (121)
			HN_1(U) $\ldots N_1$	4 (3)
N_6(C)	O_6(G)	3·05 (2·98)	$C_6N_6 \ldots O_6$	123 (125)
			$HN_6 \ldots O_6$	10 (2)
N_1(G)	N_1(C)	2·95 (2·95)	C_6N_1(G) $\ldots N_1$	122 (122)
			C_2N_1(G) $\ldots N_1$	116 (116)
			HN_1(G) $\ldots N_1$	3 (3)
N_2(G)	O_2(C)	3·07 (3·03)	$C_2N_2 \ldots O_2$	122 (123)
			$HN_2 \ldots O_2$	6 (0)

Corresponding values for the untwisted Crick-Watson base-pairs (as derived by Spencer) are in brackets. A, U, G and C indicate that the atom is in adenine, uracil, guanine and cytosine.

and two of them (C_3 out of plane on the C_5 side of the sugar and C_2 out of plane on the O_1 side of the sugar) result in O_6 coming too close to the phosphate group attached to C_3. In the RNA model O_6—C_4' is 3·01 Å. If thymine were present in RNA instead of uracil O_6 would be only 2·94 Å from the carbon atom of the 5-methyl group of the preceding nucleotide, about 0·5 Å less than the accepted minimum value.

On the average the atoms in the phosphate group of the RNA model are at a radius 0·3 Å greater than that in the DNA. This is due partly to the increase in OB (Section 4) but mainly due to the need to increase the distance between the phosphate group and O_6. The sugar-phosphate chain between C_4' and P and between P and C_3 is fully extended (Fig. 1: projection perpendicular to the helix axis) and the angles at C_5' (113°) O_4 (123°), P (104°) and O_1 (126°) are close to maximum permissible value.

It is expected that the hydroxyl group attached to the sugar C_2 will take part in a hydrogen bond. The only atoms in the same RNA molecule that could act as acceptors in this bond are the phosphate oxygens O_2, O_3 and O_4. In configurations of the DNA

B type O_3 is too far from O_6 for hydrogen bonding. Since (by analogy with DNA) O_2 and O_3 share a charge of $-e$, O_2 is more likely to be the acceptor than O_4. In the RNA model the distance O_6 to O_2 is 2·45 Å and the angle $C_6O_6O_2$ is 110°. Although this bond is intramolecular, because of the size of the DNA nucleotide, it is more analogous to the intermolecular than the intramolecular bonds which have been observed in structures of small molecules. Intermolecular O—H . . . O bonds have been observed as short as 2·45 Å, though infrequently (Fuller, 1959).

Two non-bonded interatomic distances in the sugar-phosphate chain, O_1—C_5' and O_4—C_3' (Fig. 1), are less than the normal van der Waals distance. All short non-bonded interatomic distances in the model are in Table 5. The bonds and angles in the base-pairs are as derived by Spencer (1959*a*). All the bond lengths in the sugar-phosphate chain (Fig. 1) are within 0·02 Å of accepted values. All the bond angles seem reasonable although it is difficult to define permissible limits, particularly for the external angles of the sugar ring.

Table 5

Non-bonded interatomic distances less than the normal van der Waals values

C_4 (Pyrimidine)	C_2 (Sugar)	2·94 Å
O_6 (Sugar)	O_2 (Phosphate)	2·45†
O_1 (Phosphate)	C_3' (Sugar)‡	2·87
O_4 (Phosphate)	C_5' (Sugar)‡	2·86

† Proposed hydrogen bond.
‡ ′ Indicates the atom is in the preceding nucleotide.

6. Summary of X-ray and Molecular Model Building Studies of RNA

Rich & Watson (1954*a* and *b*; see also Rich, 1957) obtained X-ray diffraction patterns from high molecular weight RNA from various sources. No detailed molecular model has been derived from these patterns. Zubay & Wilkins (1960) obtained X-ray diffraction patterns from unoriented *E. coli* microsomal RNA which were similar to those from unoriented DNA in the *B* configuration. They suggested that the RNA might have a two-stranded helical structure based on hydrogen bonding between those bases with 6-keto groups and those with 6-amino groups. Such base-pairs would not be equivalent and consequently the sugar-phosphate chain would be irregular.

It has been pointed out by Dr. M. H. F. Wilkins that there is no stereochemical reason why the extra hydroxyl group of the RNA cannot be accommodated in the model for the *A* configuration of DNA (Langridge *et al.*, 1957). Such an addition is possible whether or not thymine has been replaced by uracil. This model will be referred to as the *A* model for RNA and the model whose atomic co-ordinates are given in Table 2 as the *B* model. The diffraction patterns which have been obtained from RNA are compared in Table 6 with the diffraction which would be given by these models.

The intensity peak at about 25 Å in the observed patterns is due to the molecular packing and corresponds to sampling of the Fourier transform of a single molecule and not to a peak in this transform. The intensity distribution in the calculated *B* pattern

is similar to that observed from soluble RNA from *E. coli*. However, none of the observed patterns have the 3·3 and 4·0 Å intensity peaks with the same relative size and shape as those in the pattern calculated for the *A* model.

7. Conclusions

1. A stereochemically satisfactory model for RNA has been described which is very similar to that for the *B* configuration of DNA. This configuration would be impossible if the uracil of RNA were replaced by thymine. This is because the carbon atom of the 5-methyl group would be 0·5 Å closer than the normal van der Waals distance to the oxygen atom of the extra hydroxyl group of the ribose sugar.

TABLE 6

Comparisons of the spacings of the intensity peaks in the observed diffraction patterns from RNA with those in the diffraction patterns calculated for the models described in this paper (see Section 6)

Authors	Rich & Watson (1954*a*)		Zubay & Wilkins (1960)		Brown & Zubay (1960)		
Source	Various		*E. coli* (Ribosomes)		*E. coli* (soluble fraction)	RNA *B* model	RNA *A* model
Humidity	Dry	90%	75%	92%	92%		
Spacings in Å	10·1 m	24·3 vs 11·3 s	25·0 m, d 11·0 s, d	25·0 m, d 11·0 s, d	22·7 s, vsh 11·7 s, d	12·4 s, d	12·2 s, d
	7·0 vw	7·3 w	7·5 w, d	7·5 m, d	7·5 m, d	8·7 m, d	7·5 w, d
	5·3 m	5·4 m	5·5 w, d	5·5 vw	5·6 m, d	5·7 w, d	5·4 w, d
	4·0 m	3·9 m	4·0 w, vd	4·0 vw	4·0 w, d	4·0 w, vd	4·2 m, d
	3·2 m	3·2 s	3·3 m, d	3·3 s, d	3·3 m, sh	3·4 s, sh	3·3 w, vd

s, m, w, indicates that the peak is strong, medium or weak, and d and sh that it is diffuse or sharp; very is abbreviated to v.

2. This model is, like the DNA models, based on specific pairing between complementary chains of bases and therefore is relevant to the structure of RNA fractions which contain equimolar amounts of adenine and uracil and of guanine and cytosine (e.g. some S-RNA fractions) and to those regions of other RNA molecules where the base sequence allows specific pairing. Double stranded helical regions can be formed either by intermolecular hydrogen bonding between two RNA strands or by intramolecular hydrogen bonding between different parts of a single stranded molecule as in the model for RNA proposed by Fresco, Alberts & Doty (1960).

3. The calculated diffraction from this model is similar to that observed from *E. coli* S-RNA.

4. It has been pointed out that RNA can adopt a similar configuration to that of DNA in the *A* form. In this configuration the extra hydroxyl group of the RNA can be accommodated without either modification of the DNA model or the replacement

of thymine by uracil. As yet there is no X-ray evidence to suggest that RNA adopts such a configuration.

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REFERENCES

Brown, D. M. & Todd, A. R. (1955). In *The Nucleic Acids*, Vol. I, p. 409. New York: Academic Press.
Brown, G. L. & Zubay, G. (1960). *J. Mol. Biol.* **2**, 287.
Chargaff, E. (1955). In *The Nucleic Acids*, Vol. I, p. 307. New York: Academic Press.
Corbridge, D. E. C. (1960). *Acta Cryst.* **13**, 263.
Davies, D. R. & Corbridge, D. E. C. (1958). *Acta Cryst.* **11**, 315.
Davis, F. F., Carlucci, A. F. & Roubein, I. F. (1959). *J. Biol. Chem.* **234**, 1525.
Dunitz, J. D. & Rollett, J. S. (1956). *Acta Cryst.* **9**, 327.
Dunn, D. B., Smith, J. D. & Spahr, P. F. (1960). *J. Mol. Biol.* **2**, 113.
Fresco, J. R., Alberts, B. M. & Doty, P. (1960). *Nature*, **188**, 98.
Fuller, W. (1959). *J. Phys. Chem.* **63**, 1705.
Hamilton, L. D., Barclay, R. K., Wilkins, M. H. F., Brown, G. L., Wilson, H. R., Marvin, D. A., Ephrussi-Taylor, H. & Simmons, N. S. (1959). *J. Biophys. Biochem. Cytol.* **5**, 397.
Langridge, R., Seeds, W. E., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F. & Hamilton, L. D. (1957). *J. Biophys. Biochem. Cytol.* **3**, 767.
Langridge, R., Marvin, D. A., Seeds, W. E., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F. & Hamilton, L. D. (1960). *J. Mol. Biol.* **2**, 38.
Lipshitz, R. & Chargaff, E. (1960). *Biochim. biophys. Acta*, **42**, 545.
Monier, R., Stephenson, M. L. & Zamecnik, P. C. (1960). *Biochim. biophys. Acta*, **43**, 1.
Osawa, S. (1960*a*). *Biochim. biophys. Acta*, **42**, 244.
Osawa, S. (1960*b*). *Biochim. biophys. Acta*, **43**, 110.
Rich, A. (1957). In *Special Publications of New York Academy of Sciences* V, p. 186.
Rich, A. & Watson, J. D. (1954*a*). *Nature*, **173**, 995.
Rich, A. & Watson, J. D. (1954*b*). *Proc. Nat. Acad. Sci., Wash.* **40**, 759.
Singer, M. F. & Cantoni, G. L. (1960). *Biochim. biophys. Acta*, **39**, 182.
Spencer, M. (1959*a*). *Acta Cryst.* **12**, 59.
Spencer, M. (1959*b*). *Acta Cryst.* **12**, 66.
Watson, J. D. & Crick, F. H. C. (1953). *Nature*, **171**, 737.
Zubay, G. (1958*a*). *Nature*, **182**, 388.
Zubay, G. (1958*b*). *Nature*, **182**, 1290.
Zubay, G. & Wilkins, M. H. F. (1960). *J. Mol. Biol.* **2**, 105.

J. Mol. Biol. (1961) **3**, 185–201

Structure de l'Acide Désoxyribonucléique en Solution : Étude par Diffusion des Rayons X aux Petits Angles

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The structure of the sodium salt of DNA (DN . Na) in aqueous solution has been investigated by X-ray scattering techniques.

The gels that are formed at high DNA concentrations (15 to 60%) have been examined with a small-angle scattering camera (Luzzati & Nicolaïeff, 1959). The structure is liquid-crystalline and consists of parallel equidistant rods organized in a hexagonal two-dimensional lattice. Given the size of the lattice and the DNA concentration, it is possible to calculate the linear mass (weight per unit length) of DN . Na. The experimental results show that the linear mass is independent of concentration and is the same in pure water and in saline (1 M- and 3 M-NaCl): its value is in excellent agreement with the Crick & Watson model.

The isotropic solutions existing at low DNA concentrations (1 to 6%) have been studied with a new technique based upon the measurements of small-angle scattered intensity on an absolute scale (Luzzati, 1960). When the solute is rod-like in shape this technique gives the linear mass of the rods in addition to the radius of gyration around the axis.

In pure water the linear mass of DN . Na is the same as in the liquid-crystalline gels, and agrees very closely with the Crick & Watson model.

By analysing the effect of two salts, NaCl and NaBr, added to water in different concentrations, it has been established that the structure of DN . Na is insensitive to the amount of electrolyte and that the distribution of the ions in water is strongly disturbed in the vicinity of DNA molecules. The experimental data have been quantitatively interpreted by assuming that every DNA molecule is surrounded by a definite amount of "bound" water (about 70% of the mass of DNA) which is impenetrable to the ions.

Several conclusions have been drawn:

(*a*) DNA is rod-like in shape; the molecules are well dispersed in water, at all concentrations, and there are no associations similar to those we have discovered in nucleoproteins (Luzzati & Nicolaïeff, 1959).

(*b*) With our present knowledge of the structure of DNA, the most reasonable interpretation of our experimental observations leads to the conclusion that the *whole* of the DNA extracted from cells by the usual mild procedures has the same structure and that this structure is essentially that of the Crick & Watson model.

(*c*) The structure of DNA is independent of salt concentration. This feature seems to reflect an intrinsic stability of DNA: it is likely that the same structure is preserved inside the nuclei, where DNA is often associated with basic proteins.

(*d*) The perturbation of the distribution of the ions in the vicinity of the DNA molecules probably has some bearing on the chemical reactivity of DNA. Furthermore it is likely that the amount of "bound" water is a function of the base composition of DNA, and this may explain the correlation of the buoyancy density, measured in concentrated CsCl solution, with base composition.

1. Introduction

Depuis plusieurs années une des activités principales du Centre de Recherches sur les Macromolécules porte sur la configuration de l'ADN† en solution (Sadron, 1953, 1959): c'est dans le cadre de ces travaux que nous avons entrepris notre étude.

Jusqu'ici la structure de l'ADN a été étudiée principalement sur des fibres étirées et orientées, du moins par les techniques cristallographiques: il ne subsiste guère de doute que, dans ces conditions, la structure, soit celle proposée par Crick & Watson, du moins dans ses caractéristiques essentielles (Crick & Watson, 1954; Langridge *et al.* 1960*a*, *b*). La configuration de l'ADN en solution se prête plus difficilement à une étude directe et, bien que l'ensemble des données expérimentales soit compatible avec une structure en double hélice, analogue à celle des fibres (Doty, 1959), les preuves n'en sont pas absolument convaincantes.

Or, il importe de préciser quelle est la structure en solution, car c'est dans cet état que l'ADN se trouve dans les organismes vivants, qu'il soit seul ou associé à des protéines basiques. En outre, même si l'on peut établir que l'ADN passe des fibres en solution, et *vice versa*, sans modifications de sa structure interne, il reste à déterminer quelle fraction de l'ADN est organisée en double hélice: c'est une question à laquelle on n'a pas apporté jusqu'ici de réponse satisfaisante. Il est également intéressant d'étudier la structure de l'ADN en présence de sel, car l'action des électrolytes peut être à certains égards analogue à celle des protéines basiques, auxquelles l'ADN se trouve en général associé. On sait, par ailleurs, que les sels jouent un rôle important dans la stabilité de l'ADN, et dans son comportement en solution (Thomas, 1954; Emanuel, 1960).

Deux techniques expérimentales, que nous avons mises au point récemment, nous ont permis d'aborder ces problèmes. D'une part *l'étude des gels mésomorphes* que forme l'ADN avec l'eau, aux concentrations élevées: ces expériences peuvent fournir des renseignements sur la structure des molécules et, en particulier lorsqu'il s'agit de bâtonnets rigides, permettent d'en mesurer la masse linéaire. D'autre part *l'examen*

† ADN et DN . Na désignent respectivement l'acide désoxyribonucléique et son sel de sodium. Nous avons admis que l'ADN contient les quatre bases dans les mêmes concentrations molaires. La masse molaire du tétranucléotide (sel de sodium) est 1 323,57 et il contient 680 électrons.

s est le paramètre qui mesure les angles de diffraction: $s = \dfrac{2 \sin \theta}{\lambda}$, exprimé en Å^{-1}.

c (ou c_e) est la concentration, mesurée par le rapport entre la masse (ou le nombre d'électrons) du DN . Na et la masse (ou le nombre d'électrons) de la solution.

δ (ou ρ_0) est la densité (ou la densité électronique) du solvant exprimée en g cm^{-3} (ou él. Å^{-3}). Dans les cas où l'eau est salée, δ et ρ_0 se réfèrent à la solution saline.

$\bar{v}$ (ou ψ) est le volume spécifique partiel, c'est-à-dire le volume occupé en solution infiniment diluée par un gramme (ou un électron) de DN . Na, exprimé en cm^3 g^{-1} (ou Å^3 él.$^{-1}$). La relation entre ψ et $\bar{v}$ est: $\psi = \bar{v} \times 3{,}24$. On admettra dans la suite que le volume partiel est indépendant de la concentration.

$\dfrac{m}{l}$ (ou μ) est la masse linéaire du DN . Na, mesurée par la masse en grammes (ou le nombre d'électrons) d'un segment de bâtonnet de longueur unité. μ (él. Å^{-1}) $= \dfrac{m}{l}$ (g cm^{-1}) $3{,}09 \times 10^{15}$.

Dans le modèle de Crick & Watson, pour une séparation entre paires de bases de 3,36 Å (Langridge *et al.*, 1960*b*), $\dfrac{m}{l} = 327 \times 10^{-16}$ g cm^{-1}; $\mu = 101$ él. Å^{-1}.

R_c est le rayon de giration autour de l'axe du bâtonnet, de la différence entre la densité électronique à l'intérieur du bâtonnet et la densité électronique du solvant: R_c est exprimé en Å.

des solutions diluées et isotropes au moyen de la diffusion centrale des rayons X, mesurée à l'échelle absolue; ce procédé, dans le cas des solutions de bâtonnets, permet de déterminer la masse linéaire, en plus du rayon de giration autour de l'axe.

Dans le cas de l'ADN les domaines de concentration dans lesquels on peut utiliser ces deux techniques vont respectivement de 15 à 60%, et de 1 à 6% environ.

2. Partie Expérimentale

(a) *Préparation des échantillons. Détermination de la concentration*

Les ADN de thymus de veau et d'érythrocytes de poulet ont été préparés selon la technique de Mirsky & Pollister (1946): dans tous les échantillons le rapport azote sur phosphore N/P est compris entre 1,60 et 1,75 et le coefficient d'absorption spécifique ($\lambda = 250m\mu$) ϵ_p est compris entre 6 200 et 6 500.

Dans chaque expérience nous avons préparé l'échantillon en mélangeant des quantités déterminées de fibres d'ADN et d'eau, pure ou salée. Le contrôle de la concentration a été effectué après l'expérience de rayons X, en récupérant l'échantillon, et en déterminant par analyse chimique la teneur en phosphore. Cette opération est délicate, car les échantillons ont une faible masse: nous avons pris soin toutefois d'éviter les erreurs systématiques.

Toutes les expériences ont été effectuées à la température ambiante (20° à 27°C).

(b) *Le volume spécifique partiel*

Le volume spécifique partiel de l'ADN dans l'eau est un des paramètres dont dépend l'interprétation des expériences de diffraction des rayons X: il importe d'en connaître la valeur avec précision.

Plusieurs déterminations expérimentales ont été effectuées dans le passé, mais les différentes valeurs de $\bar{v}$ sont assez peu concordantes: elles vont, en effet, de 0,465 à 0,66 (Kahler, 1948). Cette dispersion semble être due, d'une part, à des difficultés inhérentes à la mesure des concentrations, et d'autre part à la viscosité élevée des solutions, qui rend précaires les mesures picnomètriques.

Plus récemment Doty & Zubay (1959) ont déterminé le volume spécifique partiel d'une nucléohistone; cette mesure semble être plus précise que celles obtenues avec l'ADN, car les solutions sont relativement peu visqueuses, et la composition chimique de la nucléohistone, ainsi que la concentration des solutions, ont été établies avec soin. En admettant que les volumes spécifiques partiels d'ADN et d'histone sont indépendant, connaissant $\bar{v}$ (nucléohistone) = 0,68 $\pm$ 0,01 (Doty & Zubay, 1959), $\bar{v}$ (histone) = 0,76 (Trautman & Crampton, 1958), et la composition de la nucléohistone (Doty & Zubay, 1959) (53% histone, 47% ADN), on obtient $\bar{v}$ (ADN) = 0,59 $\pm$ 0,025, et pour le sel de sodium, on peut estimer $\bar{v}$ (DN . Na) = 0,56 $\pm$ 0,03.

Langridge *et al.* (1960*a*) ont déterminé la densité du sel de lithium de l'ADN dans sa forme cristalline, et en ont déduit $\bar{v}$ (DN . Li) = 0,58. En admettant que les volumes partiels de Li et Na sont les mêmes, on peut calculer $\bar{v}$ (DN . Na) = 0,55. En fait il s'est avéré par la suite (Wilkins, Fuller, Marvin & Spencer, 1960) que les fibres contiennent une quantité non négligeable d'ion Cl: $\bar{v}$ (DN . Na) est donc un peu plus élevé que 0,55.

Une valeur bien plus précise de $\bar{v}$ pourrait être déduite des déterminations de densité de l'ADN dans les solutions concentrées de ClCs (Meselson, Stahl & Vinograd, 1957; Rolfe & Meselson, 1959; Sueoka, Marmur & Doty, 1959), si l'on pouvait admettre que la distribution du ClCs dans l'eau n'est pas perturbée au voisinage des molécules d'ADN. En fait nous montrerons plus loin que cette hypothèse n'est pas du tout justifiée.

3. Gels Concentrés

(a) *Technique*

Lorsque la concentration est suffisemment élevée, le système se présente sous forme d'un gel spontanément biréfringent dont la structure est mésomorphe; les diagrammes de diffraction contiennent dans leur région centrale ($s < 0,1$ Å^{-1}) quelques raies

(parfois assez floues), dont la position dépend de la concentration. La concentration à laquelle apparaît cette phase mésomorphe, ainsi que la finesse des raies, varie d'une préparation d'ADN à l'autre, et, pour un même échantillon, dépend de la teneur en sel du solvant. Pour étudier ces gels nous nous sommes servis d'une chambre de diffraction aux petits angles, à enregistrement photographique, selon la technique décrite ailleurs (Luzzati & Nicolaïeff, 1959).

Dans certains cas favorables l'on peut observer, dans la région centrale, jusqu'à trois raies: on constate alors que leurs espacements se suivent dans les rapports $1 : \sqrt{3} : \sqrt{4}$, ce qui est caractéristique d'une maille hexagonale à deux dimensions. En outre, puisque l'espacement des raies varie avec la concentration, le système contient une seule phase (voir Luzzati & Nicolaïeff, 1959). Ces observations indiquent que les molécules d'ADN, sous forme de bâtonnets rigides, s'organisent selon un réseau hexagonal, et qu'à toutes les concentrations la totalité du solvant est logée entre les bâtonnets.

Si on admet que les volumes partiels du DN . Na et du solvant sont indépendants de la concentration, on peut établir la loi de variation des espacements, en fonction de la concentration. Soit s_1 l'espacement correspondant à la première raie (qui est la plus intense), σ la surface de la maille élémentaire à deux dimensions; on peut écrire:

$$\sigma = \frac{2}{\sqrt{3}} s_1^{-2} \times 10^{-16} \text{ cm}^2 \tag{1}$$

$$c = \frac{\frac{m}{l}}{\frac{m}{l} + \left(\sigma - \frac{m}{l}\bar{v}\right)\delta} \tag{2}$$

En éliminant σ de (1) et (2) on obtient

$$s_1^{-2} \times 10^{-16} = \frac{\sqrt{3}}{2} \frac{m}{l} \frac{1}{\delta} [c^{-1} - 1 + \delta\bar{v}] \tag{3}$$

(3) est l'équation d'une droite (s_1^{-2} en fonction de c^{-1}) qui coupe l'axe des abscisses au point $c^{-1} = 1 - \delta\bar{v}$, dont la pente est $\frac{d(s_1^{-2})}{d(c^{-1})} = 10^{-16} \frac{\sqrt{3}}{2} \frac{m}{l} \frac{1}{\delta}$. C'est cette représentation que nous adoptons dans la suite: en principe elle permet de déterminer les valeurs de $\frac{m}{l}$ et de $\bar{v}$.†

(b) *Résultats expérimentaux*

Les expériences ont été effectuées sur de l'ADN extrait d'erythrocytes de poulet. Nous avons examiné un grand nombre d'échantillons, préparés dans l'eau pure et dans des solutions salines (ClNa, 1 M et 3 M).

Les résultats sont portés sur Fig. 1, selon la représentation s_1^{-2} en fonction de c^{-1}; lorsque les raies sont floues nous avons représenté leur demi-longueur par un trait vertical.

Les points s'alignent de manière satisfaisante sur des droites: la dispersion est due surtout aux erreurs de concentration. Dans l'eau pure nous avons obtenu des gels mésomorphes à des concentrations aussi basses que $c = 0{,}17$; à mesure que la teneur en sel augmente la limite d'existence des gels se déplace vers les hautes concentrations.

† Bernal & Fankuchen (1941) ont utilisé cette même représentation dans l'étude des gels de virus de la mosaïque du tabac, sans toutefois essayer de déterminer ni m/l ni $\bar{v}$.

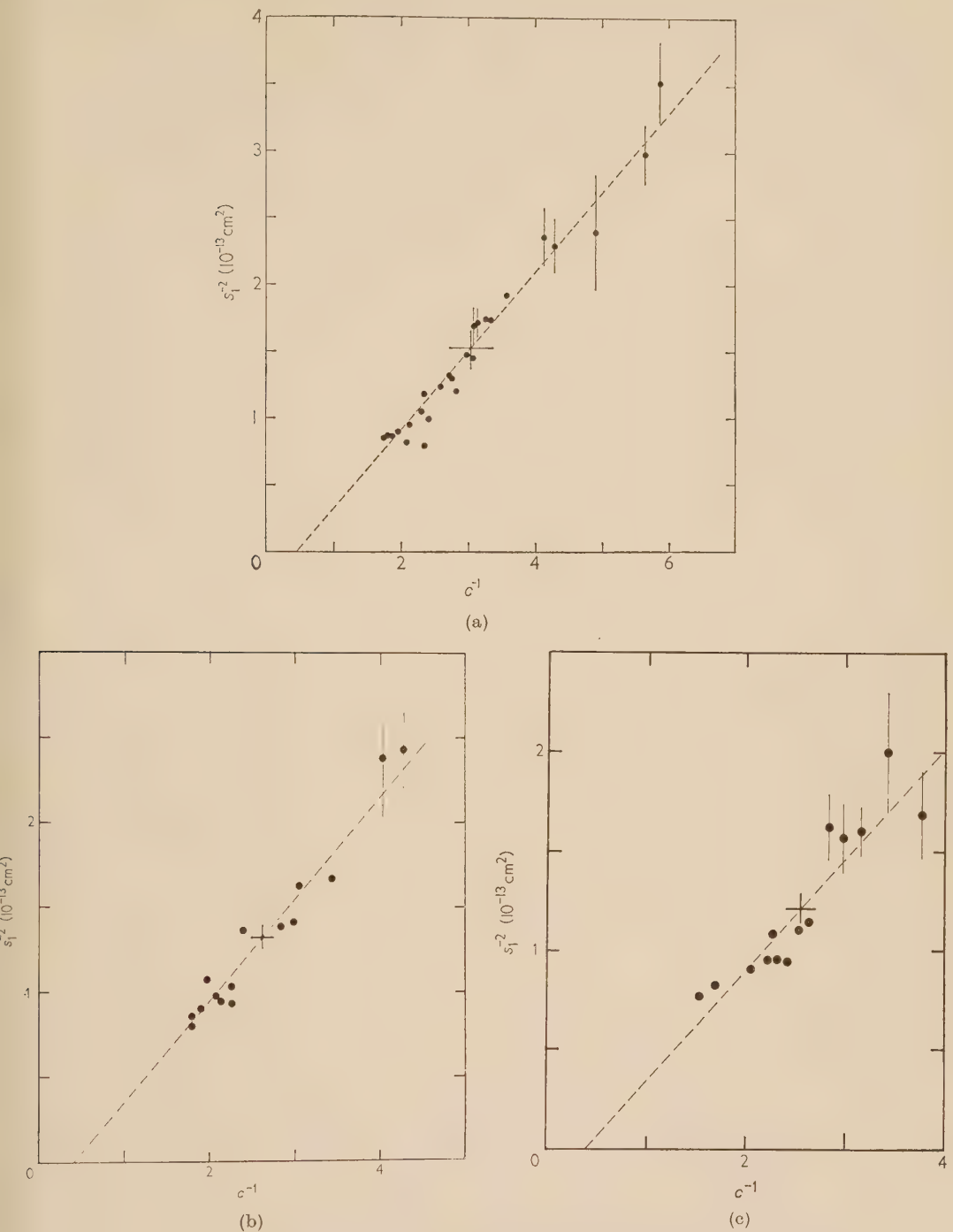


FIG. 1. Résultats expérimentaux, obtenus avec les gels mésomorphes (éq. 3). Lorsque la raie est floue, on représente sa demie largeur par un trait vertical. La position du centre de gravité des points expérimentaux est indiquée par une croix. Les droites tracées en trait interrompu correspondent à $\bar{v} = 0{,}57$.

(*a*) Expériences effectuées dans l'eau pure.
(*b*) Expériences effectuées dans l'eau salée (ClNa, 1 M).
(*c*) Expériences effectuées dans l'eau salée (ClNa, 3 M).

O

Puisque la position de la droite (éq. 3) est peu sensible à la valeur de $\bar{v}$, nous avons renoncé à déterminer $\bar{v}$ à partir des données expérimentales; nous avons préféré calculer m/l en mesurant la pente de la droite qui passe par le centre de gravité des points expérimentaux et par le point $c^{-1} = 1 - \delta\bar{v}$, et ceci pour différentes valeurs de $\bar{v}$ m/l ainsi calculé est porté dans le Tableau 1. On constate d'une parte que m/l est presque

Tableau 1

Valeurs de m/l *obtenues avec les gels mésomorphes (éq. 3 et Fig. 1):* m/l *est exprimé en unités* 10^{-16} *g cm*$^{-1}$

	Eau	Eau + ClNa 1 M	Eau + ClNa 3 M	$\overline{\left(\frac{m}{l}\right)}$
Nombre de points expérimentaux	27	15	14	
δ (g cm^{-3})	1,00	1,04	1,11	
$\frac{m}{l}$ ($\bar{v} = 0{,}50$)	350	371	369	360
$\frac{m}{l}$ ($\bar{v} = 0{,}55$)	342	362	360	352
$\frac{m}{l}$ ($\bar{v} = 0{,}60$)	337	354	351	345
$\frac{m}{l}$ ($\bar{v} = 0{,}65$)	330	348	342	338

indépendant de $\bar{v}$, et d'autre part que les valeurs obtenues dans les trois groupes d'expériences sont les mêmes, dans la limite des erreurs expérimentales. La valeur moyenne de m/l, pour les trois groupes d'expériences, est représentée, en fonction de $\bar{v}$, dans Fig. 2.

Dans les gels mésomorphes la masse linéaire de l'ADN est donc indépendante de la concentration et de la teneur en sel de l'eau: sa valeur est en outre en bon accord avec le modèle de Crick & Watson.

4. Solutions Diluées

(a) *Technique*

Aux faibles concentrations les solutions sont optiquement isotropes et les diagrammes de diffraction des rayons X ne contiennent qu'une tache diffuse qui entoure le faisceau incident.

La loi de distribution angulaire de l'intensité de cette tache est caractéristique de la forme et des dimensions des particules de soluté. En particulier, lorsqu'il s'agit de bâtonnets longs et rigides cette loi est connue (Luzzati, 1960): dans ce cas l'analyse des courbes expérimentales permet de déterminer la masse linéaire des bâtonnets, lorsque les intensités sont mesurées à l'échelle absolue, et le rayon de giration R_c autour de l'axe (voir notation), ce dernier même en l'absence de mesures absolues.

Le principe de la technique expérimentale est le suivant (voir dans Luzzati, 1960, la description détaillée). Le rayonnement X, issu d'un tube à foyer fin dont l'émission est stabilisée, est reçu par un monochromateur à lame de quartz courbée, qui focalise le faisceau monochromatique sur un segment de droite, parallèle à la fente d'entrée

d'un compteur de Geiger. Un système de fentes réglables définit et limite le faisceau incident. Un jeu de filtres soigneusement étalonnés, qui peuvent s'intercaler sur la faisceau à l'entrée du compteur de Geiger, permet d'en atténuer l'intensité dans des rapports allant jusqu'à 10^{-6}. L'échantillon enfermé dans une cuve plate, entre deux feuilles de mica, est centré sur l'axe de rotation d'un goniomètre; l'épaisseur de la cuve est mesurée à l'aide d'un double microscope, par visée des deux feuilles de mica. On effectue une expérience en enregistrant, en fonction de l'angle de diffusion, l'intensité

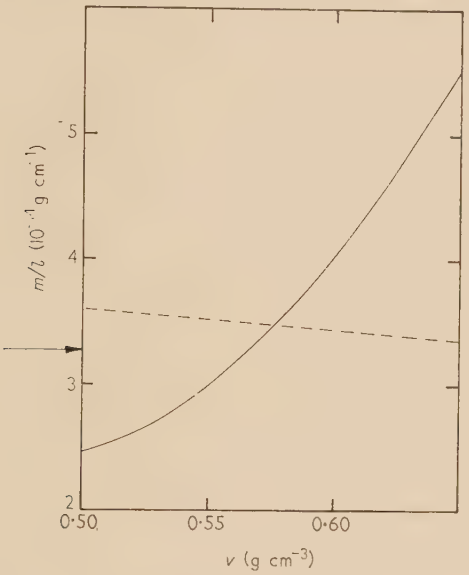


FIG. 2. Représentation de m/l en fonction de $\bar{v}$: en trait plein, courbe relative aux solutions diluées (éq. 7); en trait interrompu, courbe relative aux gels mésomorphes (Tabl. 1, éq. 3 et Fig. 1). La valeur correspondant au modèle de Crick & Watson est indiquée par une flèche.

$I_1(s)$ reçue par le compteur et intégrée, pendant la rotation continue du goniomètre, et en mesurant, dans des conditions analogues, l'énergie $\int i_0(s)\,ds$ du faisceau incident, atténué par des filtres; on soustrait de l'intensité diffusée par la solution l'intensité $I_2(s)$ convenablement normée, que diffuse la même cuve, remplie de solvant. On peut alors calculer une function expérimentale normée:

$$j_n(s) = \frac{I_1(s) - I_2(s)}{\nu\eta\int i_0(s)ds} \tag{4}$$

où η est le nombre d'électrons de l'échantillon, par centimètre carré (η dépend de la densité et de l'épaisseur de l'échantillon), ν est une constante ($\nu = \lambda^2 \times 7{,}9 \times 10^{-26}$). La fonction $j_n(s)$ ne dépend que de la structure de l'échantillon.

Dans le cas d'une solution diluée de bâtonnets, examinée par un dispositif à collimation linéaire, nous avons proposé (Luzzati, 1960) une représentation que convient pour déterminer simultanément R_c et μ. Nous avons remarqué, en effet, que du moins pour s petit, $j_n(s)$ a la forme †

$$j_n(s) = A[\tfrac{1}{2}\exp(-\pi^2 R_c^2 s^2) K_0(\pi^2 R_c^2 s^2)] \tag{5}$$

$$A = \mu c_e (1 - \rho_0\psi)^2 \tag{6}$$

† Il faudrait, en toute rigueur, tenir compte d'une correction (Luzzati, 1960)

$$j_n(s) = A\tfrac{1}{2}\exp(-\pi^2 R_c^2 s^2) K_0(\pi^2 R_c^2 s^2) + g(s)$$

En fait, dans tous les cas étudiés ici, cette correction s'est avérée négligeable.

En utilisant une représentation doublement logarithmique ($\log j_n$ en fonction de $\log s$) les courbes expérimentales se déduisent de la courbe théorique

$$\log \{A[\tfrac{1}{2} \exp (- \pi^2 R_c^2 s^2) K_0(\pi^2 R_c^2 s^2)]\}$$

par une translation sans rotation: les déplacements d'abscisses et d'ordonnées mesurent respectivement R_c et A.

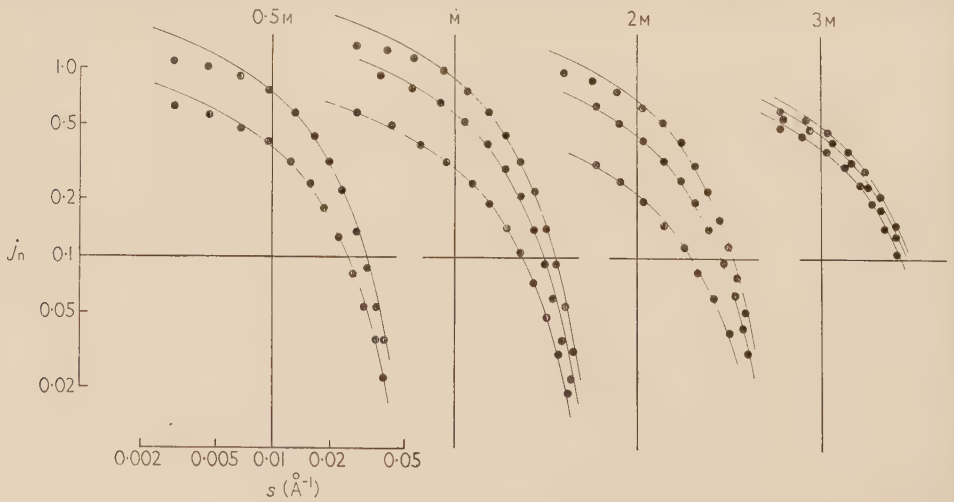
Le traitement décrit ci-dessus n'est valable qu'à des dilutions assez grandes pour les corrélations en position et orientation entre les molécules de soluté soient négligeables. Dans la pratique on peut éliminer les effets des corrélations en extrapolant à concentration nulle les résultats de mesures effectuées à différentes concentrations.

(b) *Résultats expérimentaux*

Nous avons effectué plusieurs séries d'expériences avec deux types d'ADN (de thymus de veau et d'érythrocytes de poulet) et deux sels (ClNa et BrNa): le type d'ADN, la nature et la concentration du sel sont fixes dans chaque série, la concentration d'ADN est variable. Dans tous les cas nous avons atteint des concentrations aussi faibles que le permet la sensibilité de l'appareil; naturellement cette limite inférieure varie avec la nature du sel et sa concentration, dont dépendent l'absorption massique et ρ_0 (et A (éq. 6)).

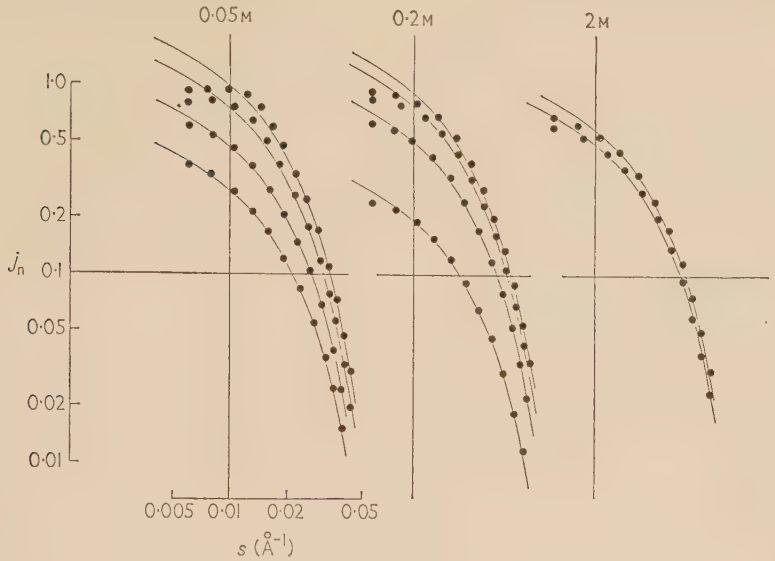
Les résultats de chaque série d'expériences sont portés sur Fig. 3, selon la représentation $\log [j_n(s)]$ en fonction de $\log s$: les points ont été relevés sur des courbes continues, à des intervalles arbitraires. Bien que les expériences n'aient pas été toutes faites dans des conditions identiques, la région de s explorée est dans tous les cas suffisemment étendue pour permettre de déterminer simultanément A et R_c.

Le choix des valeurs de A et R_c a été guidé par les considérations suivantes. Si les corrélations étaient négligeables les fonctions $j_n(s)$ de chaque série d'expériences devraient être proportionnelles à la concentration; on sait, par ailleurs, que les effets des corrélations, lorsqu'ils se manifestent, augmentent régulièrement avec la concentration, et sont d'autant plus prononcés que s est petit (Guinier & Fournet, 1955). Donc, si on porte $\log [j_n(s)/c_e]$, au lieu de $\log [j_n(s)]$ (Fig. 3), on doit obtenir une famille de courbes, qui coïncident toutes, pour s grand, et s'ouvrent régulièrement en éventail, lorsque s diminue: on peut alors éliminer l'effet des corrélations en extrapolant

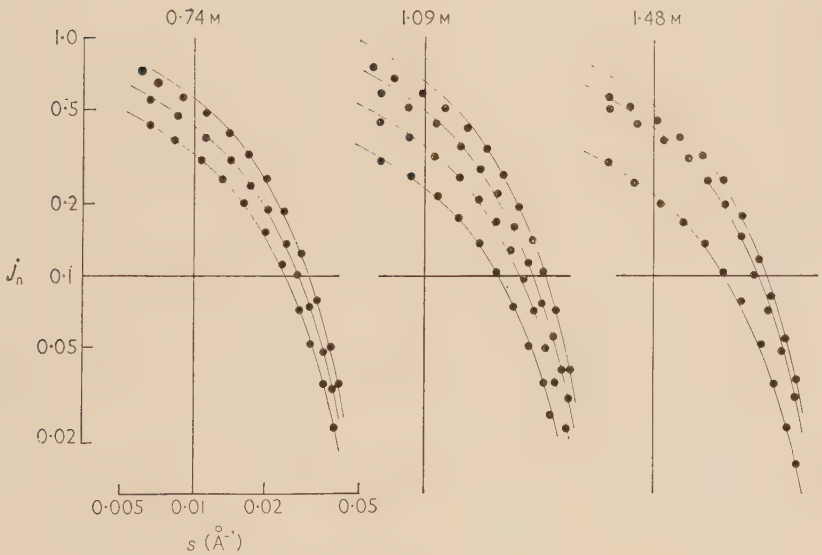


(a)

Fig. 3



(b)



(c)

FIG. 3. Représentation doublement logarithmique des courbes expérimentales $j_n(s)$ et des fonctions théoriques $\frac{A}{2}\exp(-\pi^2 R_c^2 s^2) K_0(\pi^2 R_c^2 s^2)$ correspondant à chacune des courbes expérimentales. La concentration en DN . Na, et la valeur de A et R_c, relatives à chaque expérience sont portées dans le Tableau 2.

(*a*) ADN d'erythrocytes de poulet, en solution saline (eau + NaCl): la molarité est indiquée sur la figure.

(*b*) ADN de thymus de veau, en eau + ClNa.

(*c*) ADN de thymus de veau, en eau + BrNa.

TABLEAU 2

Valeur des paramètres relatifs aux expériences effectuées avec les solutions diluées.

(a) *ADN d'erythrocytes de poulet, en solution dans l'eau + ClNa* (*Fig.* 3(a)).

Concentration saline	ρ_0 él. Å^{-3}	c %	c_e %	R_e Å	A	$\frac{A}{c_e}$	$\overline{\left(\frac{A}{c_e}\right)}$	μ_a él. Å^{-1}
0,50 M	0,3377	2,26	2,10	8,3	0,286	13,61		
0,50	0,3377	4,41	4,11	8,3	0,556	13,52	13,56	96,3
1,00 M	0,3443	1,92	1,79	8,1	0,218	12,17		
1,00	0,3443	3,72	3,47	8,1	0,421	12,13		
1,00	0,3443	5,61	5,23	8,1	0,641	12,25	12,12	92,4
2,00 M	0,3547	1,75	1,64	7,8	0,154	9,39		
2,00	0,3547	3,47	3,25	7,8	0,313	9,63		
2,00	0,3547	5,65	5,30	7,8	0,475	8,96	9,32	78,9
3,00 M	0,3645	3,99	3,77	7,6	0,267	7,08		
3,00	0,3645	4,49	4,24	7,6	0,317	7,47		
3,00	0,3645	4,95	4,68	7,6	0,351	7,50	7,35	69,3

(b) *ADN de thymus de veau, en solution dans l'eau + ClNa* (*Fig.* 3(b))

0,05 M	0,3335	1,38	1,27	8,4	0,211	16,61		
0,05	0,3335	2,50	2,31	8,4	0,357	15,45		
0,05	0,3335	4,45	4,11	8,4	0,571	13,89		
0,05	0,3335	5,15	4,76	8,4	0,746	15,67	15,40	104,9
0,20 M	0,3353	1,07	0,99	8,4	0,149	15,05		
0,20	0,3353	2,82	2,61	8,4	0,393	15,05		
0,20	0,3353	4,49	4,15	8,4	0,602	14,50		
0,20	0,3353	5,10	4,71	8,4	0,694	14,73	14,83	102,9
2,00 M	0,3547	4,00	3,75	7,8	0,363	9,68		
2,00	0,3547	4,61	4,33	7,8	0,427	9,86	9,77	82,6

(c) *ADN de thymus de veau, en solution dans l'eau + BrNa* (*Fig.* 3 (c))

0,74 M	0,3485	2,27	2,10	7,9	0,236	11,23		
0,74	0,3485	3,02	2,80	7,9	0,308	11,00		
0,74	0,3485	3,90	3,62	7,9	0,403	11,27	11,16	88,4
1,09 M	0,3552	2,23	2,09	7,8	0,169	8,08		
1,09	0,3552	3,04	2,86	7,8	0,253	8,84		
1,09	0,3552	4,27	4,01	7,8	0,357	8,90		
1,09	0,3552	5,62	5,28	7,8	0,476	9,01	8,70	74,0
1,48 M	0,3623	2,18	2,06	7,6	0,156	7,57		
1,48	0,3623	4,77	4,52	7,6	0,298	6,59		
1,48	0,3623	5,41	5,12	7,6	0,371	7,24	7,13	65,6

vers $c_e = 0$.† Si la courbe extrapolée appartient à la famille (5) on peut déterminer les valeurs de A et R_c.

Cette représentation toutefois risque d'être confuse: nous avons préféré adopter le procédé suivant. Ayant groupé les courbes relatives à une série d'expériences, nous avons cherché les fonctions (5), correspondant toutes à une même valeur de R_c qui coïncident aussi parfaitement que possible avec les courbes expérimentales, surtout aux grandes valeurs de s, et pour les faibles concentrations, et qui s'en écartent éventuellement, mais de la manière régulière décrite ci-dessus, lorsque s diminue et la concentration s'élève. Ce procédé est illustré dans Fig. 3. On peut remarquer que l'effet des corrélations, qui en général n'est pas très prononcé, s'estompe régulièrement lorsque s croît, la concentration en ADN diminue, et la teneur en sel augmente.

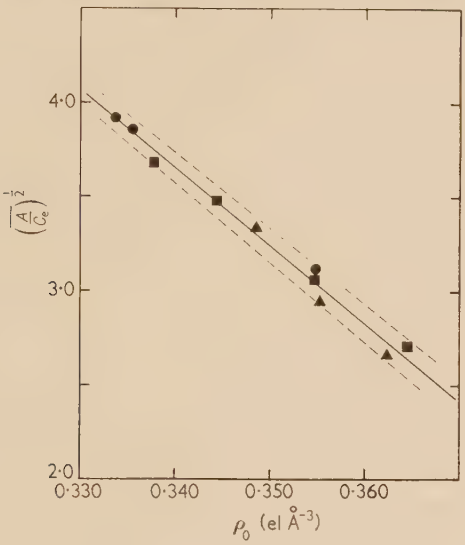


Fig. 4. Représentation $\left(\frac{A}{c_e}\right)^{\frac{1}{2}}$ en fonction de ρ_0 (Tableau 2 et éq. 11).

● ADN de thymus de veau, en solution dans l'eau + ClNa.
▲ ADN de thymus de veau, en solution dans l'eau + BrNa.
■ ADN d'érythrocytes de poulet, en solution dans l'eau + ClNa.

La zone de dispersion des points, due aux incertitudes inhérentes à l'interprétation des courbes expérimentales, est enfermée entre les lignes en pointillé (voir discussion des erreurs).

Le choix de R_c peut se faire sans une trop grande marge d'arbitraire, au prix parfois de quelques tâtonnements (voir plus loin la discussion des erreurs) : lorsque R_c est fixé les valeurs de A peuvent être déterminées avec précision. Les valeurs de R_c et A relatives aux courbes de Fig. 3 sont portées dans le Tableau 2.

5. Structure de l'ADN dans l'Eau Pure

Pour déterminer les valeurs de la masse spécifique linéaire et du rayon de giration dans l'eau il convient d'extrapoler les résultats relatifs aux solutions salines: nous n'avons pas fait d'expériences en absence de sel car d'une part l'ADN n'est pas stable

† On ignore, toutefois, la loi de variation de $j_n(s)$ avec la concentration: il n'est pas certain *a priori*, que cette loi soit linéaire, même pour c petit.

dans ces conditions (Thomas, 1954), et d'autre part le sel atténue les corrélations. On peut effectuer cette extrapolation à l'aide de la représentation $\left(\frac{A}{c_e}\right)^{\frac{1}{2}}$ en fonction de ρ_0 (Fig. 4), dans laquelle tous les points s'alignent sur une droite (les raisons de cela seront données plus loin). Pour $\rho_0 = 0{,}333$ él. Å^{-3}, correspondant à l'eau pure, $\left(\frac{A}{c_e}\right)^{\frac{1}{2}} = 3{,}94$, on a ainsi (éq. 6)

$$\mu = 15{,}50\,(1 - \rho_0\psi)^{-2}\frac{m}{l} = 50{,}2.10^{-16}\,(1 - 3{,}24\,.\,\delta\bar{v})^{-2} \qquad (7)$$

La valeur de m/l dépend du choix du volume spécifique partiel. Nous avons représenté la variation de m/l, en fonction de $\bar{v}$, dans Fig. 2, où nous avons porté la même fonction relative aux gels concentrés. Les courbes correspondant aux deux types d'expériences se croisent dans la région où $\bar{v} \cong 0{,}57$ et $m/l \cong 340 \times 10^{-16}$: ces deux valeurs sont en excellent accord d'une part avec la masse linéaire du modèle de Crick & Watson, d'autre part avec les déterminations expérimentales les plus récentes de $\bar{v}$ (voir ci-dessus).

La valeur de R_c, dans l'eau pure, est voisine de 8,4 Å (Fig. 5). Il faut rappeler que

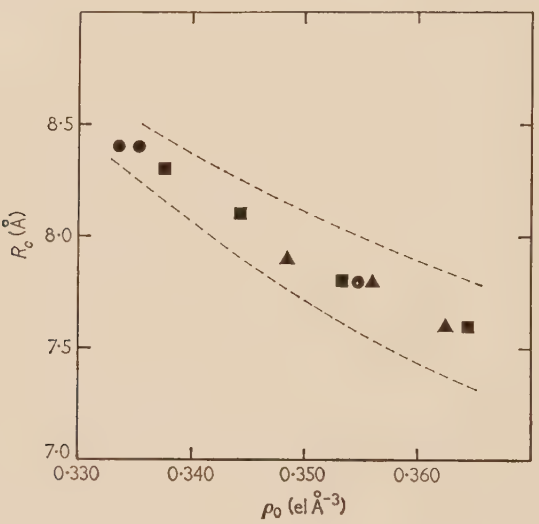


Fig. 5. La valeur de R_c, relative aux solutions diluées (Tableau 2), portée en fonction de la densité électronique du solvant.
● ADN de thymus de veau, en solution dans l'eau + ClNa.
▲ ADN de thymus de veau, en solution dans l'eau + BrNa.
■ ADN d'érythrocytes de poulet, en solution dans l'eau + ClNa.
La région d'incertitude du choix de R_c est comprise entre les deux lignes pointillées.

ce rayon de giration se réfère à la différence de densité électronique entre le DN . Na et le solvant: pour en calculer la valeur il faut donc connaître, en plus de la distance des atomes du DN . Na à l'axe de l'hélice, le nombre effectif d'électrons de chaque atome, c'est-à-dire la différence entre le nombre d'électrons de l'atome et ceux du volume d'eau que l'atome déplace. Langridge *et al.* (1960*b*) ont calculé le nombre effectif d'électrons des différents atomes de l'ADN: nous nous sommes servis de ces valeurs pour calculer R_c. Nous avons utilisé les coordonnées de quatre modèles: celui proposé par Crick & Watson (1954), et les trois discutés par Langridge *et al.* (1960*b*),

qui diffèrent du modèle de Crick & Watson surtout par un déplacement des bases vers l'axe de l'hélice. Le calcul dépend, en outre, de la distance radiale et du nombre effectif d'électrons des cations Na. Ignorant ces valeurs, nous nous sommes placés dans deux cas extrêmes : nous avons négligé, dans l'un, les cations Na, et nous avons supposé, dans l'autre, qu'il se trouvait à 12 Å de l'axe de l'hélice, et que le nombre effectif d'électrons est 11 (c'est-à-dire que le volume partiel de Na^+ est nul). Les valeurs de R_c calculées dans ces deux cas sont portées dans le tableau suivant.

Tableau 3

Modèle	R_c(Å)	R_c(Å)
	sans Na^+	avec Na^+
Crick & Watson (1954)	8,25	8,85
Langridge *et al.* (1960*b*)		
mod I	7,45	8,25
„ II	7,20	8,05
„ III	6,95	7,85
Valeur expérimentale	8,4	

L'incertitude sur le volume effectif et sur la position des cations Na^+ rend précaire le choix entre les différents modèles: nous espérons pouvoir lever ce doute en étudiant d'autres sels de l'ADN.

6. Structure et Hydratation de l'ADN dans l'Eau Salée

L'allure de la variation de $\frac{A}{c_e}$ avec la concentration saline est frappante (Fig. 4). Pour l'expliquer on peut analyser les effets de différents phénomènes:

(1) les modifications de la densité électronique du solvant;

(2) un changement éventuel de la structure de l'ADN;

(3) une perturbation possible de la distribution du sel dans l'eau, au voisinage des molécules d'ADN.

Si le troisième phénomène ne jouait aucun rôle, on pourrait obtenir la masse spécifique au moyen de la formule (6), et ceci à toute concentration en sel. Les valeurs ainsi calculées, que nous appellons μ_a dépendent fortement de la teneur en sel (Tableau 2): les variations de la densité électronique du solvant ne suffisent donc pas, à elles seules, à rendre compte des changements de $\frac{A}{c_e}$.

Si le sel modifiait la structure de l'ADN (2ème cas), les perturbations ne devraient dépendre que de la molarité des solutions salines, et ceci avec les deux sels employés, à moins d'admettre que l'un des deux anions exerce une action sélective, ce que semble être exclu (Emanuel, 1960). Or on constate (Fig. 6) que les points μ_a portés en fonction de la molarité, se groupent en deux familles distinctes, chacune correspondant à l'un des sels.

Il semble donc nécessaire d'admettre que la distribution du sel est perturbée au voisinage des molécules d'ADN. Pour effectuer une vérification quantitative de cette hypothèse il faut se référer à un modèle précis: nous avons supposé que chaque molécule d'ADN est entourée d'une couche d'eau impénétrable au sel, la structure de

l'ADN, ainsi que le volume de cette eau "liée", étant indépendants de la concentration saline.

On peut calculer facilement, dans ce cas, l'expression de $\frac{A}{c_e}$. Soient μ_h, ψ_h et $(c_e)_h$ les paramètres μ, ψ et c_e relatifs à la particule hydratée, et α le rapport entre le nombre d'électrons de cette couche d'eau "liée" et ceux du DN . Na:

$$\mu_h = \mu(1 + \alpha) \tag{8}$$

$$\psi_h = (\psi + \alpha\psi_{H_2O})(1 + \alpha)^{-1} \tag{9}$$

$$(c_e)_h = c_e(1 + \alpha) \tag{10}$$

En remplaçant dans l'équation (6), μ, ψ et c_e par les expressions (8), (9) et (10) on obtient:

$$\left(\frac{A}{c_e}\right)^{\frac{1}{2}} = \sqrt{\mu}[1 + \alpha - \rho_0(\psi + \psi_{H_2O}\alpha)] \tag{11}$$

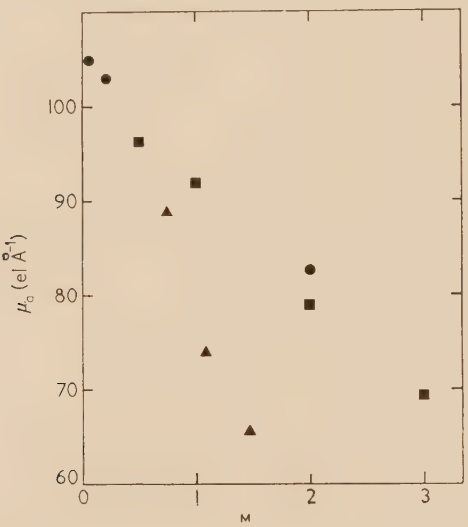


FIG. 6. Variation de μ_a (Tableau 2) avec la molarité des solutions salines.
● ADN de thymus de veau, en solution dans l'eau + ClNa.
▲ ADN de thymus de veau, en solution dans l'eau + BrNa.
■ ADN d'érythrocytes de poulet, en solution dans l'eau + ClNa.

$\left(\frac{A}{c_e}\right)^{\frac{1}{2}}$ est donc une fonction linéaire de ρ_0: or on constate (Fig. 4) que *les points expérimentaux* $\left(\frac{A}{c_e}\right)^{\frac{1}{2}}$, *qu'ils aient été obtenus avec le ClNa ou le BrNa, s'alignent sur une seule droite, lorsqu'on les porte en fonction de* ρ_0. Les paramètres de cette droite dépendent de μ (éq. 7) et de α: pour la droite représentée dans Fig. 4, α a pour expression:

$$\alpha = 3{,}51 - 1{,}504\ \psi$$

et, pour $\psi = 1{,}85$, $\alpha = 0{,}73$.

Il semble donc établi que la structure de l'ADN (ou plus exactement la forme en bâtonnet, et la masse linéaire) ne dépend pas de la concentration du sel, du moins en première approximation, et que la distribution du sel est perturbée au voisinage des molécules d'ADN. Le modèle simple d'interaction entre le sel et l'ADN utilisé dans les calculs précédents n'est pas le sel qui puisse rendre compte des observations ex-

périmentales: on pourrait obtenir également une variation linéaire de $\left(\frac{A}{c_e}\right)^{\frac{1}{2}}$ en fonction de ρ_0 si on supposait par exemple que la concentration relative des anions et des cations est perturbée au voisinage de l'ADN.

La variation de R_c avec ρ_0 (Fig. 5) peut être justifiée sans difficulté, du moins de manière qualitative; il est impossible toutefois, d'en faire un calcul exact, sans connaître la distribution de l'eau "liée" autour des molécules d'ADN.

7. Précision des Résultats

Puisque nous présentons ici les premières applications d'une technique nouvelle, il convient d'examiner d'un oeil critique les différentes sources d'erreurs.

Il n'est pas nécessaire de considérer en détail les erreurs accidentelles dues à des mesures courantes. Qu'il s'agisse de déterminations de concentration, de mesures d'épaisseur des cuves, de relevés des courbes expérimentales, ou de calculs intermédiaires, les erreurs qui affectent ces opérations ne sont pas bien importantes, et

Tableau 4

La masse de quelques protéines en solution

Protéine	Lysozyme	β-Lactoglobuline
Solvant	eau + NaCl (0,15 M)	eau + tampon acétate $\mu = 0{,}1$ pH = 5,7
Masse R.X. (1)	14 100	36 000
$\bar{v}$ (1)	0,714 (2)	0,743 (3)
Masse connue	14 700 ± 250 (4)	35 500 ± 500 (5)

(1) Masse déterminée par diffusion centrale des rayons X, mesurée à l'échelle absolue (Luzzati, 1960): on justifie le choix de $\bar{v}$, dont dépend la masse R.X.
(2) Charlwood (1957).
(3) Timosheff, communication personnelle.
(4) Fromageot & Privat de Garilhe (1950).
(5) Green & Aschaffenburg (1959).

surtout n'ont pas de caractère systématique: on peut donc en atténuer les effets en multipliant le nombre des expériences. On peut estimer l'importance de ces erreurs par les écarts entre les différentes valeurs de $\frac{A}{c_e}$ (voir Tableau 2), qui en l'absence d'erreurs, devraient être constantes dans chaque série d'expériences: la dispersion moyenne des points, autour de la valeur moyenne est d'environ 2,5%.

Bien plus graves, parce que systématiques, peuvent être les effets d'erreurs sur les coefficients d'absorption des filtres. Ces coefficients ont été mesurés avec soin, par un procédé qui sera décrit ailleurs: dans nos mesures, où les coefficients d'absorption sont compris entre 1×10^4 et 7×10^4, on peut estimer que l'erreur qui les affecte est inférieure à 5%.

Une vérification indépendante de la précision de la technique expérimentale dans son ensemble est fournie par les résultats obtenus avec des solutions de protéines: la

diffusion centrale absolue permet, dans ce cas, de déterminer la masse moléculaire (Luzzati, 1960). Ces valeurs des masses sont portées dans le Tableau 4, et sont comparées à celles déterminées par d'autres techniques expérimentales: l'accord est excellent.

Une autre source d'erreurs est inhérente à la technique d'interprétation des courbes $j_n(s)$. En effet le choix de R_c est souvent incertain, à cause d'une part des erreurs expérimentales, qui s'aggravent aux concentrations faibles, et d'autre part des effets des corrélations qui déforment les courbes aux concentrations élevées. Les valeurs de R_c sont toutefois comprises entre les deux lignes pointillées de Fig. 5. Le domaine de $\left(\frac{A}{c_e}\right)^{\frac{1}{2}}$, correspondant aux valeurs extrêmes de R_c, est limité par les deux lignes tracées en pointillé dans Fig. 4. Les valeurs limités de m/l et α (pour $\psi = 1{,}85$) sont: $324 \times 10^{-16} < m/l < 357 \times 10^{-16}$, $0{,}6 < \alpha < 0{,}9$.

8. Discussion et Conclusions

L'ensemble des résultats indique que la structure de l'ADN est, à toute concentration, celle d'un bâtonnet long et rigide, et que la dispersion des molécules dans le solvant est complète, sans qu'il se forme d'aggrégats ou d'édifices complexes, analogues à ceux que nous avons observés dans les solutions de nucléoprotéines (Luzzati & Nicolaïeff, 1959).

Par l'interprétation des expériences effectuées avec les gels mésomorphes nous avons déterminé la masse linéaire des bâtonnets en solution concentrée (15 à 60%), dans l'eau pure et salée (ClNa, 1 M et 3 M). La valeur de la masse linéaire qu'on obtient en étudiant les solutions diluées et isotropes dépend fortement de la valeur du volume spécifique (Fig. 2): pour $\bar{v} = 0{,}57$, qui correspond aux plus récentes déterminations expérimentales, la masse linéaire en solution diluée est identique à celle des gels mésomorphes. Puisque ces deux valeurs sont en excellent accord avec le modèle de Crick & Watson une conclusion semble s'imposer: *au moins le* 90% *sinon la totalité de l'ADN extrait des cellules adopte en solution la structure proposée par Crick & Watson, et ceci à toute concentration.* Cette interprétation, dérivée de mesures de masse linéaire et de rayon de giration, n'est pas, en toute rigueur, la seule qui soit compatible avec l'ensemble des données expérimentales: c'est la plus simple, et, dans l'état actuel des connaissances sur la structure de l'ADN (voir Crick, 1957), la plus vraisemblable.

Les expériences effectuées en présence de sel ont mis en évidence certaines propriétés de l'ADN. Ainsi la structure s'est avérée indépendante de la concentration saline, du moins pour le ClNa entre 0,05 et 3 M, et le BrNa jusqu'à 1,5 M. Cette stabilité semble être une propriété intrensèque de l'ADN, qui subsiste vraisemblablement à l'intérieur des cellules vivantes: l'ADN conserve probablement la même structure au cours des modifications du milieu environnant, et en particulier lorsqu'il est associé à des protéines basiques.

D'autre part la distribution du sel, dans l'eau, est fortement perturbée au voisinage des molécules d'ADN. Nous avons interprété nos expériences par la présence d'une couche d'eau "liée" impénétrable au sel, dont la masse est environ 0,75 de celle du DN . Na: cette quantité est en fait assez petite, à peine plus grande que celle nécessaire pour combler les deux sillons de la double hélice. Cette perturbation du solvant pourrait avoir des conséquences sur la réactivité de certains agents chimiques sur l'ADN, et pourrait en outre expliquer la variation de la densité, en fonction de la

composition en bases, qu'on a observée dans les solutions concentrées de ClCs (Rolfe & Meselson, 1959; Sueoka *et al.*, 1959).

Bien que nous n'ayons utilisé jusqu'ici que deux types d'ADN (de thymus de veau et d'érythrocytes de poulet), il y a tout lieu de croire (Hamilton *et al.*, 1959) que les résultats obtenus puissent être généralisés à d'autres types d'ADN, sauf bien entendu, le taux d'hydratation, qui dépend probablement de la composition en bases. C'est d'ailleurs un problème dont nous nous occuperons dans un mémoire ultérieur.

Nous tenons à remercier les collègues du Service de Biologie du C.R.M., et tout spécialement M. J. Pouyet, qui ont bien voulu se charger de la préparation de l'ADN ainsi que des analyses chimiques des échantillons.

En outre nous devons à l'obligeance de MM. S. Timasheff et J. Witz de pouvoir faire état de mesures de masses de protéines, effectuées au moyen de la diffusion centrale absolue des rayons X, qui n'ont pas encore été publiées.

REFERENCES

Bernal, J. D. & Fankuchen, I. (1941). *J. Gen. Physiol.* **25**, 111.
Charlwood, P. A. (1957). *J. Amer. Chem. Soc.* **79**, 776.
Crick, F. H. C. (1957). Dans *The Chemical Basis of Heredity*, edité par W. D. McElroy & B. Glass. Baltimore: Johns Hopkins University Press.
Crick, F. H. C. & Watson, J. D. (1954). *Proc. Roy. Soc.* A, **223**, 80.
Doty, P. (1959). *Rev. Mod. Phys.* **31**, 107.
Doty, P. & Zubay, G. (1959). *J. Mol. Biol.* **1**, 1.
Emanuel, C. F. (1960). *Biochim. biophys. Acta*, **42**, 91.
Framageot, G. & Privat de Garilhe, M. (1950). *Biochim. biophys. Acta*, **4**, 509.
Green, D. W. & Aschaffenburg, R. (1959). *J. Mol. Biol.* **1**, 54.
Guinier, A. & Fournet, G. (1955). Dans *Small Angle Scattering of X-Rays*, p. 40. New York: Wiley & Sons.
Hamilton, L. D., Barclay, R. K., Wilkins, M. H. F., Brown, G. L., Wilson, H. R., Marvin, D. A., Ephrussi-Taylor, H. & Simmons, N. S. (1959). *J. Biophys. Biochem. Cytol.* **5**, 397.
Kahler, H. (1948). *J. Phys. Coll. Chem.* **52**, 676.
Langridge, R., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F. & Hamilton, L. D. (1960*a*). *J. Mol. Biol.* **2**, 19.
Langridge, R., Marvin, D. A., Seeds, W. E., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F. & Hamilton, L. D. (1960*b*). *J. Mol. Biol.* **2**, 38.
Luzzati, V. (1960). *Acta Cryst.* **13**, 939.
Luzzati, V. & Nicolaïeff, A. (1959). *J. Mol. Biol.* **1**, 127.
Meselson, M., Stahl, F. W. & Vinograd, J. (1957). *Proc. Nat. Acad. Sci., Wash.* **43**, 581.
Mirsky, A. E. & Pollister, A. W. (1946). *J. Gen. Physiol.* **36**, 117.
Rolfe, F. & Meselson, M. (1959). *Proc. Nat. Acad. Sci., Wash.* **45**, 1039.
Sadron, C. (1953). *Progr. Biophys.* **3**, 237.
Sadron, C. (1959). *Les Nucléoprotéines*, IIème Conseil de Chemie Solvay. Interscience.
Sueoka, N., Marmur, J. & Doty, P. (1959). *Nature*, **183**, 1429.
Thomas, R. (1954). *Biochim. biophys. Acta*, **14**, 231.
Trautman, R. & Crampton, C. F. (1958). *J. Amer. Chem. Soc.* **81**, 4036.
Wilkins, M. H. F., Fuller, W., Marvin, D. A. & Spencer, M. (1960). Communication au 5ème Congrès International de Cristallographie, Cambridge.

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Irreversible Changes of Ionic Activities following Thermal Denaturation of Sodium Deoxyribonucleate

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The degree of binding of sodium ions by the macro-ion in DNA solutions has been investigated following thermal treatments at different temperatures. A sharp irreversible increase in the activity of Na^+ ions has been found to occur in a very narrow range of temperature where similar discontinuous changes both of optical rotation and of optical density had been previously observed. In consideration of the polyelectrolytic character of DNA, and with the aid of a simple theoretical approach, the above effect has been explained in terms of a decrease of charge density of the DNA macro-ion upon denaturation due to the presence of short sequences of "stretched" conformations in the coiled DNA molecule.

1. Introduction

In a previous investigation (Ascoli, Botré, Crescenzi, Mele & Liquori, 1959*a*) it was shown that the process of disordering of the DNA brought about by lowering the ionic strength of the solution below a critical range is marked by the occurrence of a discontinuity in the plot of the activity coefficient of sodium counter-ions as a function of dilution. Such an effect was ascribed to a co-operative decrease of the charge density in the DNA molecule and therefore of the electrical potential acting on the counter-ions during dilution denaturation (Liquori, Ascoli, Botré, Crescenzi & Mele, 1958).

From the very extensive studies of Doty and his school on the large irreversible changes of optical rotation, viscosity and u.v. absorption accompanying heat denaturation of DNA (Doty, Boedtker, Fresco, Haselkorn & Litt 1959), one can infer that the degree of disordering accompanying this transition should be higher than that occurring in dilution denaturation and, therefore, that more marked changes of charge density should be expected.

The present investigation was carried out with the aim of studying such an effect quantitatively by trying to detect changes of the activity coefficient of Na^+ ions following exposure of DNA solutions to different temperatures.

2. Experimental

A number of DNA samples, partly prepared according to Kay, Simmons & Dounce (1952) and others, supplied by the Nutritional Biochemical Co., were examined. In all cases the samples were dialysed, freeze-dried and then analysed.

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The thermal treatments were performed by placing the DNA solutions in dark, sealed tubes and allowing them to stand for 1 hr in a thermostat at the desired temperature, which was controlled within 1°C. Some treatments were carried out in the presence of $3{\cdot}92 \times 10^{-3}$ M-phosphate buffer or $1{\cdot}0 \times 10^{-2}$ M-NaCl. After treatment, the solutions were allowed to cool and the ionic activities, the optical rotation, the extinction at 260 mμ and the pH were measured.

The pNas of the solutions were determined at a single DNA concentration, well above the critical concentration of dilution denaturation (Ascoli *et al.*, 1959*a*), at 20°C by means of a Beckman cationic glass electrode 78137 V, showing a very high degree of selectivity toward sodium ions. This electrode was used in conjunction with a Beckman pH meter, model G, and a calomel electrode with an agar bridge by simply replacing the glass electrode used for pH measurements.

A calibration curve was prepared by stepwise dilution of a NaCl solution and recording at each step the e.m.f. of the cell. A linear plot of the e.m.f. against log a^{Na^+} is shown in Fig. 1. The pNas of the DNA solutions were obtained from the measured e.m.f.s by inter-

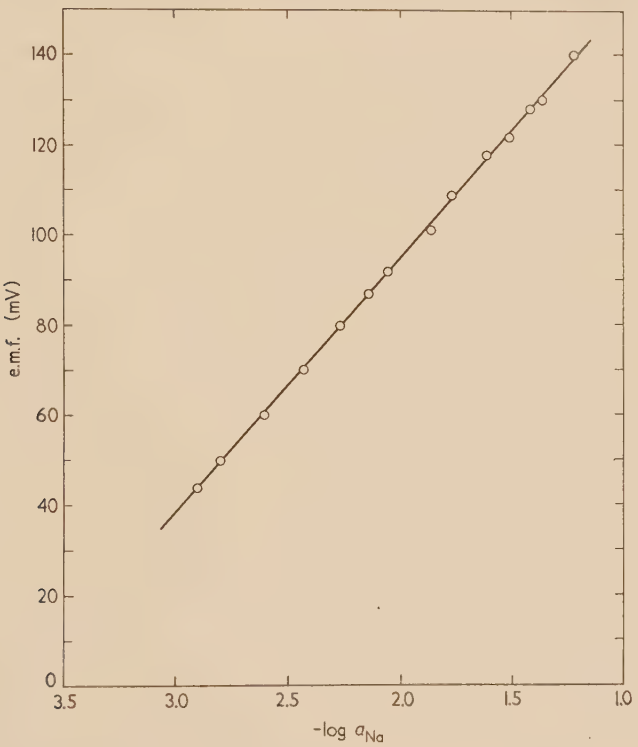

FIG. 1. Calibration curve of the Beckman glass electrode with respect to sodium ions.

polating the calibration curve. The activity coefficients were easily computed from the known value of the DNA concentration expressed in phosphorus equivalents. The optical rotations were measured at room temperature using a Galileo polarimeter equipped with a sodium lamp.

3. Results and Discussion

In Fig. 2 the pNas measured at 20°C after treatment of the DNA solutions at different temperatures are plotted against the temperature together with the corresponding values of the activity coefficients of the Na^+ ions.†

† The approximations involved in deriving the activity coefficients of single ions from membran e potential measurements have been discussed by Botré, Crescenzi, Liquori & Mele (1959). In the

A sharp increase of the activity coefficient in a very narrow temperature range is evident from Fig. 2.

That this effect is genuinely associated with heat denaturation is confirmed by:

(a) the occurrence of a critical change in the same temperature range in the presence of buffer (though in this case the effect is damped by the contribution of the sodium ions of the buffer to the pNa of the solution) and

(b) the comparison with the critical changes in optical rotation and optical density at 260 mμ observed for the same solutions in the same very narrow temperature range. This can be seen in Fig. 3.

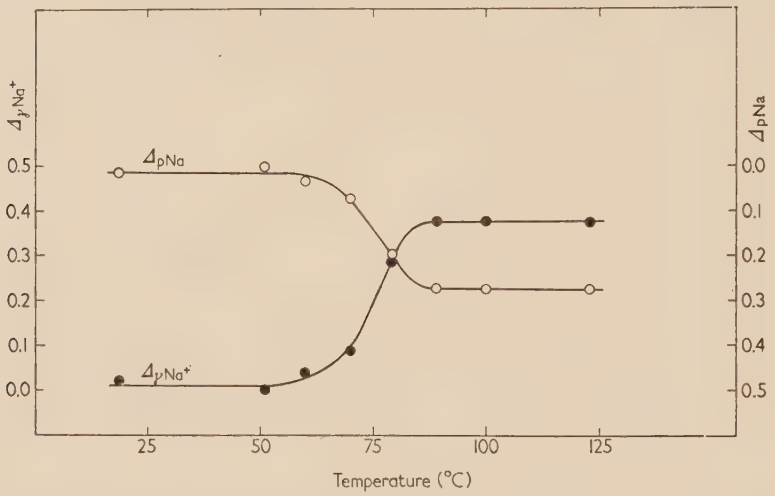

FIG. 2. ΔpNa and $\Delta\gamma Na^+$ of a DNA solution ($6{\cdot}47 \times 10^{-3}$ M) measured at 20°C as a function of the temperature of the previous heat treatment. The changes are referred to the values of the untreated solution.

From the above results it may be safely concluded that the strong binding of sodium counter-ions to the macro-ions in the DNA solution—which is typical of highly charged anionic polyelectrolytes (Ascoli, Botré, Crescenzi, Liquori & Mele, 1959*b*)—undergoes a strikingly large reduction upon heat denaturation of the molecule.

Recent determinations of the activity coefficient of counter-ions in solutions of linear polyelectrolytes with different structures (Ascoli *et al.*, 1959*b*; Kagawa & Katsuura, 1955; Nagasawa & Kagawa, 1957) have emphasized the simple connection between this quantity and the spacing of ionizable groups along the chain. Such a relation agrees with both the exact and the approximate solutions of the Poisson-Boltzmann equation, obtained on the assumption that the polyion can be considered as an infinite cylinder with a fixed radius and regularly spaced electrical charges (Fuoss, Katchalsky & Lipson, 1951; Alfrey, Bery & Morawetz, 1951; Oosawa, 1957).

present case, the influence of small pH changes has been neglected in spite of the value of the transference number of Na^+ and H^+ through the membrane being close to unity, since the pNas were smaller than the pHs (~ 6·7 to 7·0) by several decades. Using the relation

$$\Delta E = \frac{RT}{F} \ln \frac{(a_{Na^+} + a_{H^+})i}{(a_{Na^+} + a_{H^+})e}$$

for the membrane potential difference across the glass membrane, it may be shown that the above approximation does not introduce appreciable error. (See *Ion Transport across Membranes*, Academic Press, 1954, and "Membrane Phenomena", *Disc. Faraday Soc.* **21**, 1956.)

Furthermore, a negligible influence of either the length or the radius of the cylinder appears to be consistent both with the experimental results and with theoretical expectation (Ascoli *et al.*, 1959*b*; Alfrey *et al.*, 1951; Oosawa, 1957).

It may be instructive to use an approximate relation derived by Oosawa (1957) in order to estimate what change the spacing between ionizable groups of the DNA double helix, considered as a charged cylinder in solution, should undergo in order to account for the observed increase of the degree of dissociation of the counter-ions.

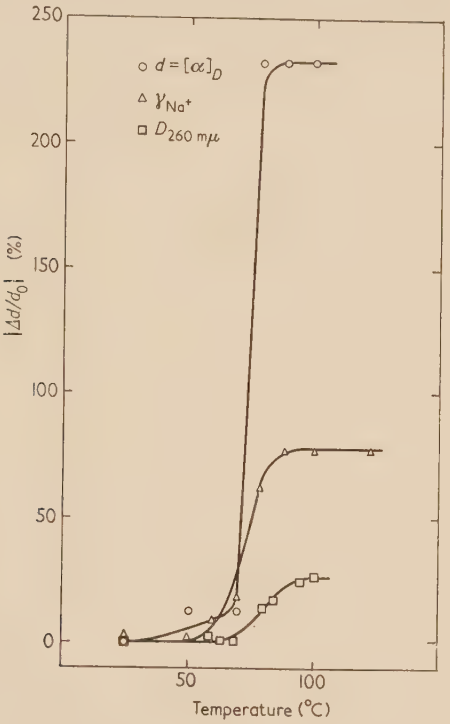

FIG. 3. Absolute changes of specific rotation, Na^+ activity coefficient and optical density measured at 20°C as a function of the temperature of heat treatments. The changes are referred to the values of the untreated solution.

In Fig. 4 a plot of the spacing b against the degree of dissociation α has been calculated with the aid of Oosawa's (1957) relation for a concentration of $6{\cdot}5 \times 10^{-3}$ M-DNA, and for cylinder radii of 20 Å and 30 Å. As may be seen, the function is rather insensitive to the choice of the radius.

From this plot it would appear that an increase in the repeat from about 3·5 Å to about 7·5 Å would be consistent with the observed increase of the Na^+ activity coefficient following heat denaturation. This might be taken as evidence for a "stretching" of the repeat upon denaturation from the value characteristic of the Crick & Watson (1953) helix to that corresponding to an almost fully extended conformation.

At first sight, such an explanation might appear in striking contrast with the description of heat denaturation of DNA in terms of a transition from a tight double helix into a completely random coil (Doty *et al.*, 1959) as inferred mainly from the reduction of the molecule in solution.

However, this inconsistency might be considered as only an apparent one by assuming that, besides the well-established double-stranded helix of DNA (Crick & Watson, 1953), other conformations are possible with varying degrees of stability.

Considering that the Crick-Watson helix has a rather high electrical free-energy due to the close contacts between the phosphate groups, and that it must be largely balanced by the decrease of free-energy corresponding to the unidimensional crystallization of the paired bases, one would expect that "the melting of the bases" induced by temperature during heat denaturation should contribute to the relative stability of conformations with a longer repeat of the ionizable groups.

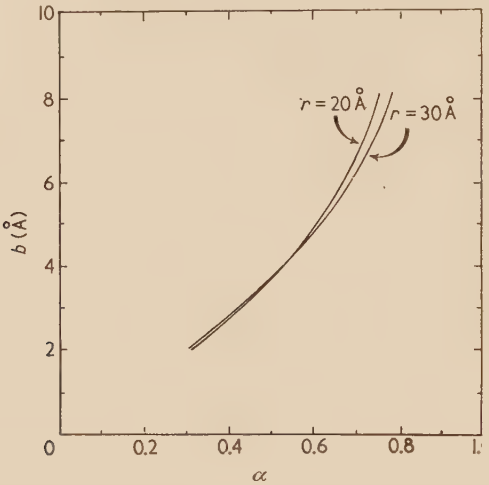

FIG. 4. Spacing b of ionizable groups along the DNA helix as a function of the degree of dissociation of the sodium ions, calculated with Oosawa's (1957) relation (for an infinite cylinder), for two different values of the radius.

The large decrease of counter-ion binding occurring on denaturation indicates that conformations having a repeat corresponding to the almost fully extended DNA chains are highly populated in the denaturated DNA molecule. If these conformations can form relatively short sections, connected through segments acting as joints, the experimental observations can be accounted for by assuming that the helix-coil transition of DNA is accompanied not only by a decrease of the radius of gyration of the whole molecule, but also by an increase of its contour length.

The reduced vertical interaction between the bases in denatured DNA would also help to explain the observed changes in the optical rotation (Fitts & Kirkwood, 1956 *a*, *b*) and in the u.v. absorption (Michelson, 1958).

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REFERENCES

Alfrey, T., Bery, P. V. & Morawetz, H. (1951). *J. Polymer Sci.* **7**, 543.
Ascoli, F., Botré, C., Crescenzi, V., Mele, A. & Liquori, A. M. (1959*a*). *Nature*, **184**, 1482.
Ascoli, F., Botré, C., Crescenzi, V., Liquori, A. M. & Mele, A. (1959*b*). *J. Polymer Sci.* **40**, 169.

Botré, C., Crescenzi, V., Liquori, A. M. & Mele, A. (1959). *Trans. Faraday Soc.* **55**, 1975.
Crick, F. H. C. & Watson, J. D. (1953). *Nature*, **171**, 737.
Doty, P., Boedtker, H., Fresco, J. R., Haselkorn, R. & Litt, M. (1959). *Proc. Nat. Acad. Sci., Wash.* **45**, 482.
Fitts, D. D. & Kirkwood, J. G. (1956*a*). *Proc. Nat. Acad. Sci., Wash.* **42**, 33.
Fitts, D. D. & Kirkwood, J. G. (1956*b*). *J. Amer. Chem. Soc.* **78**, 2650.
Fuoss, R. M., Katchalsky, A. & Lipson, S. (1951). *Proc. Nat. Acad. Sci., Wash.* **37**, 579.
Kagawa, I. & Katsuura, K. (1955). *J. Polymer Sci.* **17**, 365.
Kay, E. R. M., Simmons, N. S. & Dounce, A. L. (1952). *J. Amer. Chem. Soc.* **74**, 1724.
Liquori, A. M., Ascoli, F., Botré, C., Crescenzi, V. & Mele, A. (1958). III*rd International Symposium on Electrolytes*, Trieste. In the press.
Michelson, A. M. (1958). *Nature*, **182**, 1502.
Nagasawa, N. & Kagawa, I. (1957). *J. Polymer Sci.* **25**, 61.
Oosawa, F. (1957). *J. Polymer Sci.* **23**, 421.

J. Mol. Biol. (1961) **3**, 208–218

A Procedure for the Isolation of Deoxyribonucleic Acid from Micro-organisms†

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A method has been described for the isolation of DNA from micro-organisms which yields stable, biologically active, highly polymerized preparations relatively free from protein and RNA. Alternative methods of cell disruption and DNA isolation have been described and compared. DNA capable of transforming homologous strains has been used to test various steps in the procedure and preparations have been obtained possessing high specific activities. Representative samples have been characterized for their thermal stability and sedimentation behaviour.

1. Introduction

To facilitate the study of the biological, chemical and physical properties of DNA it is necessary to obtain the material in a native, highly polymerized state. Several procedures have described the isolation of DNA from selected groups of micro-organisms (Hotchkiss, 1957; Zamenhof, Reiner, DeGiovanni & Rich, 1956; Chargaff, 1955). However, no detailed account is available for the isolation of DNA from a diverse group of micro-organisms. The reason for this is that micro-organisms vary greatly in the ease with which their cell walls can be disrupted, in their content of capsular polysaccharides (which are difficult to separate from DNA) and in the association of DNA to protein which influences the ease of DNA purification (Kirby, 1957). Most of these difficulties have been overcome in the present procedure which has been applied successfully to approximately 50 different species of micro-organisms. Included in this number are those organisms whose DNA can transform homologous and closely related strains and that have thus provided a very useful tool in determining the efficacy of many of the steps outlined in the procedure.

In general, the method to be described can be outlined as follows: the cells are first disrupted, the cell debris and protein removed by denaturation and centrifugation, the RNA removed by RNase and the selective precipitation of the DNA with *iso*-propanol. Degradation by DNase and divalent metal ion contamination is prevented by the presence of chelating agents and by the action of sodium lauryl sulfate. The products obtained, although they may not reflect the *in vivo* molecular weight, have molecular weights in excess of 6×10^6 and a high specific transforming activity, where this is present.

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2. Materials and Methods

(a) *Reagents*

Saline-EDTA, 0·15 M-NaCl plus 0·1 M-ethylenediaminetetra-acetate (EDTA), pH 8. The EDTA, and/or high pH, inhibit DNase activity.

Sodium lauryl sulfate, 25%. The anionic detergent ($NaC_{12}H_{26}SO_4$) will lyse most non-metabolizing cells, inhibit enzyme action and denature some proteins (Bayliss, 1937; Bolle & Kellenberger, 1958).

Lysozyme, crystalline (Armour). Used to lyse cells resistant to detergent action. Cells lysed with lysozyme are then subjected to sodium lauryl sulfate as well.

Sodium perchlorate, 5 M. The high salt concentration provided by the perchlorate (Lerman & Tolmach, 1957) helps dissociate protein from nucleic acid.

Chloroform-isoamyl alcohol, 24 : 1 (v/v). Used to deproteinize, according to the method of Sevag, Lackmann & Smolens (1938). The chloroform causes surface denaturation of proteins. The *iso*amyl alcohol reduces foaming, aids the separation, and maintains the stability, of the layers of the centrifuged, deproteinized solution.

Ethyl alcohol, 95%. Used to precipitate nucleic acids following deproteinization. Denatured ethyl alcohol may also be used.

Saline-citrate, 0·15 M-NaCl plus 0·015 M-trisodium citrate, pH 7·0 ± 0·2. Maintains ionic strength of dissolved DNA and chelates divalent ions.

Dilute saline-citrate, 0·015 M-NaCl plus 0·0015 M-trisodium citrate. DNA dissolves more readily in dilute salt solutions but should *never* be dissolved in pure water.

Concentrated saline-citrate, 1·5 M-NaCl plus 0·15 M-trisodium citrate. The concentrated solution is used to bring the dilute saline-citrate solute, in which the nucleic acid is dissolved, up to saline-citrate concentration. The volume added need only be approximate until the final pure product is obtained.

Ribonuclease, 0·2% (crystalline, Armour) in 0·15 M-NaCl, pH 5·0. The solution is heated at 80°C for 10 min to inactivate any contaminating DNase. The RNase digests the RNA and facilitates its separation from DNA.

Acetate-EDTA, 3·0 M-sodium acetate plus 0·001 M-EDTA, pH 7·0. This provides the proper ionic environment in the *iso*propanol step for the separation of DNA from RNA or its digestion products (Simmons, personal communication).

Iso*propanol*. Used to precipitate DNA selectively; RNA remains in solution. In some cases it will selectively precipitate and separate DNA from polysaccharides.

(b) *Equipment for DNA isolation*

Centrifuges, Servall SS-1 operating at 5 to 10,000 rev/min (3,000 to 13,000 ***g***) and a clinical swinging bucket centrifuge capable of spinning at 2 to 3000 rev/min (300 to 600 ***g***).

Glass-stoppered flasks, for deproteinization.

Shaker, wrist action or reciprocal for deproteinization. Several hundred strokes/min.

Volumetric pipette, 10 to 15 ml. fitted with an 18 in. (approx.) rubber tube attached to the upper end. Used to remove the aqueous layer from the deproteinized, centrifuged mixture.

Stirring motor, fitted with a glass stirring rod with a screw taper. Used to stir the solution (500 to 1,000 rev/min) during the *iso*propanol addition.

(c) *Physical and biological measurements*

Determination of T_m. The method has been previously described (Doty, Boedtker, Fresco, Haselkorn & Litt, 1959; Marmur & Doty, 1959).

Determination of sedimentation coefficient and molecular weight. The sedimentation coefficient, $S_{20,w}$, of DNA dissolved in standard saline-citrate was determined in the Spinco ultracentrifuge model E at a concentration of 20 μg/ml. at a speed of 35,600 rev/min using ultraviolet optics. The centrifuge cell was fitted with a Kel-F centerpiece. The molecular weight of the sample can then be obtained using the relationship established by Doty, McGill & Rice (1958):

$$S_{20,w} = 0{\cdot}063\, M_w^{0.37}$$

Transformation. The transformation of *Diplococcus pneumoniae* was carried out by the the method of Fox & Hotchkiss (1957) using transformable, glycerol-treated, cells stored at −20°C. When it was found necessary to examine the biological properties of *Bacillus subtilis* DNA, the method of Spizizen (1959) was used to transform this organism.

(d) *Isolation procedure*

The procedure is designed for 2 to 3 g wet packed cells. The volumes are only approximate unless otherwise stated. All operations can be performed at room temperature except the RNase treatment, which is carried out at 37°C.

Bacteria grown to the logarithmic phase of their growth cycle are harvested by centrifugation and washed once with 50 ml. saline-EDTA. After collecting by centrifugation, the cells are suspended in a total volume of 25 ml. of saline-EDTA. Lysis [1,2,3],† is effected by the addition of 2·0 ml. sodium lauryl sulfate and the mixture placed in a 60°C[4] water bath for 10 min then cooled to room temperature. Lysis of the culture results in a dramatic increase in viscosity[5] accompanying the release of the nucleic acid components and some clearing. If, on preliminary testing the cells are insensitive to the detergent, but sensitive to lysozyme, approximately 10 mg of lysozyme are added to the cells suspended in saline-EDTA. The mixture is then incubated at 37°C with occasional shaking and the lysis followed by noting the increase in viscosity. In some cases 30 to 60 min may be required for optimum results. When lysozyme is used sodium lauryl sulfate is added as well, *after* the cells have lysed, followed by the 60°C heating and cooling.

Perchlorate is added to a final concentration of 1 M to the viscous, lysed suspension and the whole mixture shaken with an equal volume of chloroform-*iso*amyl alcohol in a ground-glass stoppered flask for 30 min.[6] The resulting emulsion[7] is separated into 3 layers by a 5-min centrifugation at 5,000 to 10,000 rev/min in the Servall. The upper aqueous phase contains the nucleic acids and is carefully pipetted off into a tube or narrow flask. The nucleic acids are precipitated by gently layering approximately 2 vol ethyl alcohol on the aqueous phase. When these layers are gently mixed with a stirring rod, the nucleic acids "spool" on the rod as a threadlike precipitate[8] and are easily removed. The precipitate is drained free of excess alcohol by pressing the spooled rod against the vessel. The precipitate is then transferred to approximately 10 to 15 ml. of dilute saline-citrate[9] and gently removed from the stirring rod by swirling it back and forth. The solution is gently shaken or pipetted until dispersion is complete (lumps can be recognized by adhering air bubbles when the solution is shaken). The solution is adjusted approximately to standard saline-citrate concentration by adding concentrated saline-citrate, shaken as before with an equal volume of chloroform-*iso*amyl alcohol for 15 min, centrifuged[10] and the supernatant removed. It is then deproteinized repeatedly with chloroform-*iso*amyl alcohol,[11] as described, until very little protein is seen at the interface.

The supernatant obtained after the last in the series of deproteinizations, is precipitated with ethyl alcohol and dispersed in saline-citrate (about 0·5 to 0·75 the supernatant volume) in the manner already described. Ribonuclease[12] is added to a final concentration of 50 μg/ml. and the mixture incubated for 30 min at 37°C. Following the digestion of the RNA it becomes possible to remove protein which resisted earlier chloroform deproteinizations. The digest is again subjected to a series of deproteinizations until there is little or no denatured protein visible at the interface

† Numbers refer to the following section on Procedure notes.

after centrifugation. The supernatant, after the last such treatment, is again precipitated with ethyl alcohol and the drained nucleic acid dissolved in 9·0 ml. dilute saline-citrate. When solution has occurred, 1·0 ml. acetate-EDTA is added and while the solution is rapidly stirred, 0·54 vol[13] *iso*propyl alcohol is added dropwise into the vortex. The DNA usually precipitates in a fibrous form after first going through a gel phase at about 0·5 vol *iso*propyl alcohol. RNA or oligoribonucleotides and cellular or capsular polysaccharides remain behind, while the DNA threads wind around the glass propeller. If the yield is good, the DNA is redissolved and precipitated once more with *iso*propanol in the manner described. The final precipitate is washed free of acetate and salt by gently stirring the adhered precipitate in progressively increasing (70 to 95%) portions of ethyl alcohol, and is then dissolved in the solvent of choice. If the solution is not clear, it can be clarified by centrifuging in the Servall centrifuge for 10 min at 5,000 rev/min.[14]

By using caution and recovery steps, up to 50% of the DNA from the cell is obtained; in general 1 to 2 mg of DNA is obtained from 1 g wet packed cells.[15] The DNA can be stored in solution at 5°C in the presence of several drops of chloroform.[16,17,18] If it is so desired, the DNA (free of spores) can be sterilized by exposure to 75% ethyl alcohol for several hours and then transferred to a sterile solvent.

Notes on the procedure for the isolation of DNA

It would be very difficult to describe a definitive technique for the efficient isolation of DNA from a wide variety of micro-organisms. The method described can undoubtedly be modified or improved to eliminate difficulties encountered with specific strains. In general, the Gram negative organisms yield themselves readily to the procedure resulting in good recoveries of highly polymerized DNA. Several suggestions are offered to improve yields and eliminate some of the difficulties that may arise.

1. A spot test on a centrifuged portion of the culture should be made to determine whether the organisms are susceptible to sodium lauryl sulfate, lysozyme or neither of the two. If lysed by both detergent and enzyme, the former is preferable since DNase is inactivated in its presence. If lysozyme is used, the detergent is added *after* maximum enzymatic lysis is attained.

2. Organisms that are readily lysed by sodium lauryl sulfate include:— all Gram negative strains thus far encountered (Enterobacteriaceae, *Hemophilus influenzae*, *Rhizobium Japonicum*, *Pseudomonas aeruginosa*, *Pasteurella pestis*) as well as *D. pneumoniae* (which also lyses readily with deoxycholate) *Bacillus stearothermophilus*, *B. macerans*, *B. brevis*, *B. licheniformis*, *Clostridium madisoni*, *Cl. Chauvei*, *Cl. butylicum*, *Rhodospirillum rubrum*, *Micrococcus lysodeikticus* and *Mycoplasma* (PPLO). *Euglena gracilis* and *Chlamydomonas reinhardii* and the slime mold *Dictyostelium discoideum* are readily lysed by the detergent. Some strains of *Streptococcus* and of *M. pyogenes* var *aureus* lyse slowly with sodium lauryl sulfate and cell disruption is sometimes facilitated by raising the temperature of the lysing mixtures to between 70° and 75°C. The former genus can also be lysed by extracts from *Streptomyces albus* (McCarty, 1952).

The organisms lysed with lysozyme include: *B. subtilis*, *B. natto*, *B. cereus*, *B. megaterium*, *Cl. perfringens* as well as the Actinomycetes (*S. albus* and *S. viridochromogenes*) which lyse very slowly with the enzyme (Sohler, Romano & Nickerson, 1958), depending on the state of growth when harvested.

It has been found that some strains of *Streptococcus* and of *M. pyogenes* var *aureus*,

Lactobacillus acidophilus and baker's yeast are insensitive to lysis by either lysozyme or detergent. Yeast cells can be lysed by an extract from the snail *Helix pomatia* (Eddy & Williamson, 1957). If no means of enzyme or detergent lysis is available, the cells can be disrupted by grinding with alumina or glass powder (see below). The product, however, usually has a lower molecular weight than that obtained by the other methods described. (For other means of cell wall disruption see review by Weibull, 1958.)

The method has been successfully employed in the isolation of DNA from animal tissue, using sodium lauryl sulfate to lyse the cells.

3. It has been noted that some cells grown in the presence of 5-bromodeoxyuridine have altered their susceptibility to lysis. Thus, *D. pneumoniae* grown in the presence of this analogue will lyse slowly if at all with sodium lauryl sulfate but remains susceptible to deoxycholate. This situation has not arisen in the case of *E. coli*.

4. If potent nuclease action is anticipated (e.g. *Serratia marcescens*), the detergent lysed suspension should be heated to within 10°C of the DNA T_m to eliminate its activity.

5. Some organisms (e.g. *Klebsiella pneumoniae*) are difficult to harvest and when lysed give rise to extremely viscous solutions. This is due to polysaccharide at which is usually eliminated in the final stages of the DNA isolation but may also be removed earlier by initial use of the *iso*propanol step.

6. The shaking in the first deproteinization is carried out for 30 min because of the high viscosity of the lysate; subsequent steps are for 15 min.

7. A very critical step in the procedure is the concentration of cells being lysed. Too low a cell concentration will give rise to losses in subsequent alcohol precipitation whereas, if the cell suspension is too thick, the first chloroform deproteinization will result in a "lumpy" emulsion. The lumps consist of denatured protein with large amounts of occluded DNA and form a voluminous middle layer when the mixture is centrifuged. This difficulty is easily remedied in subsequent preparations by lysing a more dilute suspension of cells. In order to recover DNA from the denatured protein layer, because of excessive occlusion or when a high yield of DNA is desired under normal circumstances, the residue is shaken with a 10 ml. portion of dilute saline citrate for 15 min, centrifuged and the precipitate obtained from the ethyl alcohol addition combined with the remainder of the preparation.

8. If difficulty is encountered in collecting a majority of the nucleic acid threads following ethyl alcohol addition or if no threads appear because of DNA degradation, the solution is subjected to a short centrifugation, the sediment dissolved in a small volume of dilute saline-citrate and the alcohol precipitate added to the remaining portion of the preparation.

9. When dissolving the fibrous nucleic acid precipitate it is well to keep the concentration of DNA at a level of about 0·2 to 0·8 mg per ml. Too low a concentration results in degradation (Hershey & Burgi, 1960) and loss of biological activity during handling. Higher concentrations are highly viscous and difficult to handle and disperse. In the early stages of the preparation, the redissolved precipitate gives rise to turbid suspensions; as the purification proceeds, the nucleic acid takes on a glassy appearance when being dispersed and results in clear solutions.

10. If the emulsion separates readily into two phases after shaking, centrifugation in a swinging bucket clinical centrifuge at 2 to 3000 rev/min is sufficient to clear the aqueous layer of chloroform and most of the denatured protein.

11. The best time to interrupt the procedure, if it should be necessary, is after any of the deproteinization steps. In this case store the uncentrifuged emulsion until the procedure can be resumed. The average length of time required to isolate purified DNA starting from the lysis of the cells is approximately 5 to 8 hr.

12. It is also possible to isolate RNA by omitting the RNase treatment, recovering the ethyl alcohol precipitates by centrifugation and saving the solution after the DNA is removed by the *iso*propanol step. It would however be best to carry out the *iso*-propanol step earlier in the procedure.

13. Several cases have been encountered where the *iso*propanol step does not precipitate the DNA when 0·54 vol have been added (*C. reinhardii* and in one case of DNA isolated from tobacco leaves). When this occurs, larger volumes of *iso*propanol should be added. At times the precipitate is granular and can be collected by centrifugation.

14. It has been repeatedly observed that the purified DNA isolated from some spore formers (e.g. *B. brevis*, *B. subtilis*, etc.) is contaminated with viable spores. The number of spores can be reduced or eliminated by harvesting the cells when spore formation is at a minimum, centrifuging the purified DNA for about 10 min at 10,000 rev/min in the Servall and/or treatment of the DNA with phenol (see 17).

15. The method can be applied to bacterial harvests of the order of 100 g but, to avoid the awkwardness of handling large volumes, the amount of reagents used can be scaled up less than proportionately.

16. If protein removal is incomplete, storage of the DNA solution in the presence of chloroform will sometimes leave a halo of denatured protein surrounding the interface of the solutions. The protein can be removed by centrifugation.

17. The introduction of the phenol method (Kirby, 1957) to isolate DNA prompted its use on purified DNA. Purified *D. pneumoniae* DNA shaken with water saturated phenol remained undegraded and did not show any apparent loss in biological activity. Trypsin and chymotrypsin likewise had little or no effect on the molecular weight or biological activity.

18. The amino acid content (other than glycine) of the purified DNA on acid hydrolysis is approximately 0·3 to 0·5% (determined by Dr. H. Van Vunakis on the Spackman, Stein & Moore (1958) automatic amino acid analyser). Typical ratios for absorption of the DNA at 260 : 230 : 280 mμ are 1·0 : 0·450 : 0·515.

(e) *Alternative methods of cell disruption and lyophilization*

Mechanical disruption

Various techniques have been described (Gunsalus, 1957) for the mechanical disruption of cells for the isolation of enzymes, nucleic acids, etc. An attempt was made to disrupt *D. pneumoniae* cells by grinding with glass powder as well as by sonic treatment and to isolate the DNA from the disrupted cells according to the method described above. Since these and other methods might be applied to cells that resist enzyme and detergent action, we have applied them to *D. pneumoniae* as a basis of comparison to the detergent method of cell lysis.

Grinding with glass or alumina (particle size 500 mesh) is carried out at 5°C. The harvested, washed cells (with saline EDTA) are placed in a pre-cooled mortar, an equal weight of glass powder (Fisher Scientific Co.) added and the mixture ground with a pestle for 5 to 10 min. Ten vols of cold saline-EDTA, containing 2% sodium lauryl sulfate, are added and the suspension centrifuged to remove glass and large cell

debris. The supernatant is then treated in the manner described above for the isolation of DNA.

Sonic treatment is also carried out in the cold at 5°C. Saline-EDTA washed cells are suspended in 10 vol saline-EDTA and placed in the cup of the sonic apparatus (Raytheon). The suspension is exposed to 9 kc (50 w) sound waves for 5 to 30 min. Sodium lauryl sulfate is then added, the suspension centrifuged to remove any cell debris and then subjected to the isolation procedure. If the DNA does not precipitate as threads upon ethyl alcohol addition, the nucleic acids are collected by centrifugation.

Lyophilization of cells

Lyophilization is a commonly used laboratory technique for the preservation of cells. It was thought of interest to examine the DNA isolated from *D. pneumoniae* which had been subjected to freeze drying. The method employed for the isolation of DNA from the lyophilized cells is the same as that described using sodium lauryl sulfate as the lysing agent.

(f) *Isolation of DNA by cesium chloride density gradient centrifugation*

The introduction of the cesium chloride density gradient technique by Meselson, Stahl & Vinograd (1957) has made it possible to separate RNA, DNA and protein under mild conditions. By selecting the proper density and conditions of centrifugation, the RNA collects at the bottom of the centrifuge tube, the protein floats on the top and the DNA bands at or near the center. Biologically active DNA can readily be located by assaying the transforming activity of various fractions. The technique can also be adapted to separate DNA samples differing in base composition (and thus in buoyant density).

As applied to the isolation of DNA from cell lysates, the following outline has been used. A concentrated cell suspension of *D. pneumoniae* is lysed by sodium lauryl sulfate and the mixture shaken with a concentrated solution of CsCl containing 0·005 M-tris buffer (2-amino-2-hydroxymethylpropane-1 : 3-diol) plus 0·005 M-EDTA, pH 8·0. After adjusting the density to 1·706 $\pm$ 0·002 g/ml., the mixture is centrifuged in lusteroid tubes in the model L Spinco preparative centrifuge at 30,000 rev/min in the SW-39 rotor at room temperature for 3 days. When the rotor head has coasted (unbraked) to a stop, the tubes are removed, secured firmly in a vertical position and a small hole bored in the bottom of the tube with a small-gauge needle. The collected fractions, when sufficiently diluted to eliminate the inhibitory effects of CsCl on transformation (1 : 200 final dilution in the case of *D. pneumoniae*), are then assayed for biological activity. The fractions showing peak activity are combined, dialysed and treated with RNase to purify further the DNA. In one experiment, the DNA thus isolated accounted for 80% of the ultraviolet absorbing material at 260 mμ.

3. Results

A representative group of DNA samples isolated from bacteria by either lysozyme or detergent cell rupture is listed in Table 1. The sedimentation coefficient (at 20 μg/ml.) and T_m values have been determined and recorded. (The sedimentation coefficient was the same regardless of whether the centrifuge cells—regular or synthetic boundary —were filled slowly with a syringe or a wide-tipped pipette.) Even though the organisms listed vary widely taxonomically as well as in the base composition of their DNA, the purified DNA samples have approximately the same sedimentation coefficients with

PLATE I. Ultracentrifuge sedimentation pattern of *E. freundii* DNA, 0·15 M-NaCl + 0·015 M-Na citrate. Using ultraviolet optics, pictures were taken every 4 min at 35,600 rev/min. First exposure at left. DNA concentration = 20 ug/ml.: 30 mm cell with Kel-F centerpiece. The sedimentation coefficient $S_{20,w} = 29{\cdot}4$.

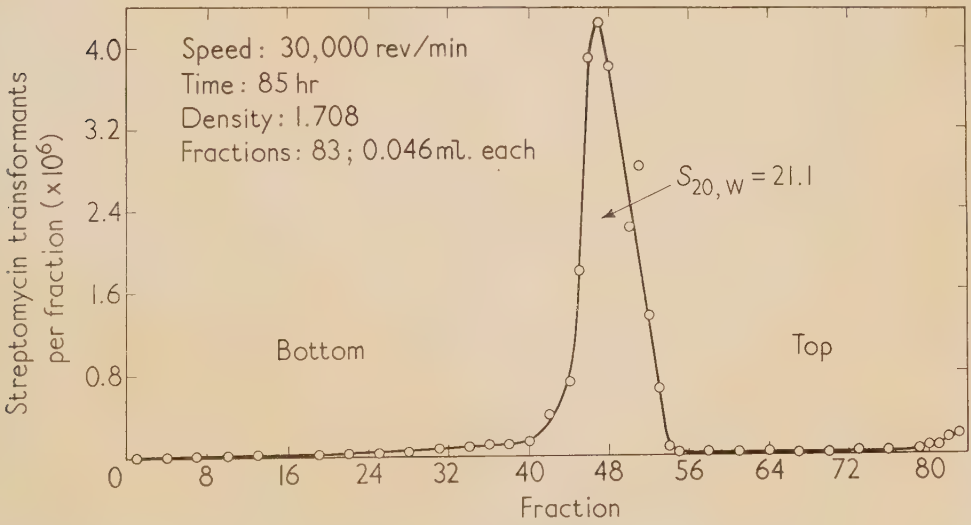

FIG. 1. CsCl density gradient isolation of DNA from streptomycin resistant *D. pneumoniae*. The ability to transform to streptomycin resistance is plotted as a function of the sample obtained by collecting drops from the punctured, lusteroid tube after centrifugation.

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calculated molecular weights of the order of 8 to 12 million. Calf thymus and salmon sperm DNAs, isolated and supplied by Dr. N. Simmons, are also listed for comparison. In determining the T_m values, the temperature-absorbance curves showed that all the DNA samples isolated were predominantly in the native configuration. Variations of the sedimentation coefficient and the T_m of different preparations of DNA from the same organism were of the order of $\pm$ 1 s and $\pm$ 0·3°C, respectively. An example of the sedimentation pattern of DNA isolated by the present method is shown in Plate I.

TABLE 1

Properties of representative DNA samples isolated from cells sensitive to lysozyme and/or sodium lauryl sulfate

Organism	$S_{20,w}$	T_m
Aerobacter aerogenes	22·4	94·0
Bacillus brevis	25·3	87·1
B. cereus	24·2	82·7
B. megaterium	29·0	85·2
B. subtilis	26·0	87·4
Diplococcus pneumoniae	24·0	85·2
Escherichia coli (K12)	29·0	90·0
Hemophilus influenzae	28·5	85·5
Klebsiella pneumoniae	25·6	92·0
Micrococcus lysodeikticus	24·0	98·6
M. pyogenes var *aureus*	28·0	83·2
Pseudomonas aeruginosa	28·2	96·7
Salmonella typhimurium	24·3	91·1
Serratia marcescens	23·7	93·6
Shigella dysenteriae	22·7	90·0
Streptococcus salivarius	24·4	85·2
Streptomyces albus	25·0	100·1
Calf thymus	22·1	86·0
Salmon sperm	23·2	87·0

The sedimentation coefficient and T_m were determined using saline-citrate (0·15 M-NaCl plus 0·015 M-Na citrate) as the solvent. The T_m for *S. albus* was obtained at a lower ionic strength and corrected to that for saline-citrate solvent. The sedimentation coefficients, determined immediately or within a day after the DNA preparation, did not alter on prolonged storage.

The effects of mechanical disruption and lyophilization of cells on the transforming activity and molecular weight of *D. pneumoniae* DNA are compared to detergent lysis in Table 2. Cells that have been subjected to freeze drying yield DNA with no detectable differences from that isolated from freshly harvested cells. However, as might be expected, sonic treatment (Litt, Marmur, Ephrussi-Taylor & Doty, 1958) has a deleterious effect on DNA. Grinding with glass is less destructive than sonic treatment and would thus be the preferred method of mechanical disruption. Other methods applying a shear force (Davison, 1959; Hershey & Burgi, 1960; Cavalieri & Rosenberg, 1959) would probably result in degradation of the DNA.

The result of the cesium chloride density gradient centrifugation technique for the isolation of DNA from *D. pneumoniae* is shown in Fig. 1. The fractions, assayed for

transformation with respect to streptomycin resistance, exhibit a peak activity at a density of CsCl expected from the base composition of the DNA (Sueoka, Marmur & Doty, 1959). The pooled active fractions, freed of contaminating RNA as described previously, had a high specific transforming activity and a molecular weight of 7×10^6 (estimated from the sedimentation coefficient). It is possible that some shear degradation of the DNA took place during mixing the lysate with CsCl, fraction collection through the narrow orifice or syringing the purified DNA into the sedimentation velocity cell (Davison, 1959). Similar experiments carried out by Mr. C. Schildkraut and Mr. R. Rownd (unpublished) employing milder conditions have yielded DNA from

Table 2

Effect of method of cell disruption and lyophilization on the transforming activity and molecular weight of D. pneumoniae *DNA*

Method of cell disruption	Relative transforming activity	Molecular weight $\times 10^{-6}$†
SLS‡	100%§	9·6
Grind with glass	36	5·0
Sonic 5′	4·1	1·0
Sonic 30′	0·42	0·38
Lyophilize, SLS	108	10·5

† Estimated from the sedimentation coefficient.
‡ SLS = sodium lauryl sulfate.
§ 100% represents $3{\cdot}6 \times 10^6$ transformants to streptomycin resistance per μg transforming factor.

E. coli with slightly higher sedimentation coefficients, in fact very similar to that for DNA isolated by deproteinization and alcohol precipitation as described. If links do exist between DNA molecules, they are disrupted by even the mildest conditions. Since the buoyant densities of the DNA in CsCl isolated by both methods are identical, one can conclude that both methods yield a product which contains the same amount of protein.

Several DNA samples were used to see whether they would transform homologous strains with respect to the streptomycin marker. Preliminary studies have shown that *E. coli* (K12), *E. freundii*, *M. pyogenes* var *aureus*. *Alcaligenes fecalis* and *Ps. aeruginosa* are incapable (less than 1 in 10^7) of being transformed, using techniques similar to those used for *D. pneumoniae* and *B. subtilis*.

4. Discussion

Micro-organisms possess DNAs that vary widely in base composition and thus offer an ideal source of this nucleic acid in the study of differences imposed on the molecule when the base pairs adenine : thymine and guanine : cytosine are present in different proportions. By studying the differences in the properties of DNA from different species, it is possible to gain an insight into the heterogeneity of the molecules comprising the genome of an organism.

The method described for the isolation of DNA has anticipated most of the difficulties that may be encountered with various micro-organisms. In general, the method described has yielded DNA from a variety of micro-organisms which is native, highly polymerized and possesses a fairly uniform molecular weight distribution. It might be argued that the method used for the isolation of DNA resulted in a degradation of the molecules and thus does not represent the true *in vivo* value. Degradation could have taken place during the shaking (Hershey & Burgi, 1960) used for deproteinization. The initial lysate is much more viscous than the final product; if the viscosity is due to a continuous DNA structure without protein links, then degradation has most likely taken place. The genetic evidence does indicate that the genome of *E. coli* behaves as one linkage group (Jacob & Wollman, 1958); however definitive evidence is still lacking as to whether this "chromosome" consists of large subunits or molecules (Forro & Wertheimer, 1960). Kellenberger (1960) has described a model in which protein "linkers" hold the DNA molecules together in the bacterial nucleoid.

The present method yields DNA from micro-organisms with a molecular weight of about 8 to 12×10^6 which is perfectly adequate for most experimental purposes. Those DNAs that possess transforming activity (e.g. *D. pneumoniae*, *B. subtilis*, etc.) can transform their homologous strains with an efficiency of 1 to 5% at saturating levels of DNA.

DNA isolated by selective buoyancy in a CsCl density gradient has physical, chemical and biological properties similar to the product obtained by the method described which involves selective denaturation with chloroform and alcohol precipitations. However, the DNA is still subjected to mild shear forces during the CsCl method and it is again possible that degradation may have taken place.

The methods of DNA isolation described in the literature, and here, are continually being improved and undoubtedly agents will be uncovered that will selectively precipitate or fractionate DNA under milder conditions than heretofore employed. The method presented in this report has been useful in providing samples of DNA from micro-organisms with widely varying base ratios and has proved of great value in studying their physico-chemical and genetic properties.

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REFERENCES

Bayliss, M. (1937). *J. Lab. Clin. Med.* **22**, 700.
Bolle, A. & Kellenberger, E. (1958). *Schweiz. Z. Path. Bakt.* **21**, 714.
Cavalieri, L. F. & Rosenberg, B. H. (1959). *J. Amer. Chem. Soc.* **81**, 5136.
Chargaff, E. (1955). In *The Nucleic Acids*, ed. by E. Chargaff & J. N. Davidson, p. 308. New York: Academic Press.
Davison, P. F. (1959). *Proc. Nat. Acad. Sci., Wash.* **45**, 1560.
Doty, P., Boedtker, H., Fresco, J. R., Haselkorn, R. & Litt, M. (1959). *Proc. Nat. Acad. Sci., Wash.* **45**, 482.
Doty, P., McGill, B. B. & Rice, S. A. (1958). *Proc. Nat. Acad. Sci., Wash.* **44**, 432.
Eddy, A. A. & Williamson, D. H. (1957). *Nature*, **179**, 1252.
Forro, F. & Wertheimer, S. A. (1960). *Biochim. biophys. Acta*, **40**, 9.

Fox, M. S. & Hotchkiss, R. D. (1957). *Nature*, **179**, 1322.
Gunsalus, I. C. (1957). In *Methods of Enzymology*, ed. by S. P. Colowick & N. O. Kaplan, Vol. 1, p. 51. New York: Academic Press.
Hershey, A. D. & Burgi, E. (1960). *J. Mol. Biol.* **2**, 143.
Hotchkiss, R. D. (1957). In *Methods in Enzymology*, ed. by S. P. Colowick & N. O. Kaplan, Vol. 3, p. 692. New York: Academic Press.
Jacob, F. & Wollman, E. L. (1958). *Symp. Soc. Exp. Biol.* **12**, 75.
Kellenberger, E. (1960). *Symp. Soc. Gen. Microbiol.* **10**, 39.
Kirby, K. S. (1957). *Biochim. J.* **66**, 495.
Kirby, K. S. (1959). *Biochim. biophys. Acta*, **36**, 117.
Lerman, L. S. & Tolmach, L. J. (1957). *Biochim. biophys. Acta*, **26**, 68.
Litt, M., Marmur, J., Ephrussi-Taylor, H. & Doty, P. (1958). *Proc. Nat. Acad. Sci., Wash.* **44**, 144.
Marmur, J. & Doty, P. (1959). *Nature*, **183**, 1427.
McCarty, M. (1952). *J. Exp. Med.* **96**, 555.
Meselson, M., Stahl, F. W. & Vinograd, J. (1957). *Proc. Nat. Acad. Sci., Wash.* **42**, 581.
Sevag, M. G., Lackman, D. B. & Smolens, J. (1938). *J. Biol. Chem.* **124**, 425.
Sohler, A., Romano, A. H. & Nickerson, W. J. (1958). *J. Bact.* **75**, 283.
Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
Spizizen, J. (1959). *Fed. Proc.* **18**, 957.
Sueoka, N., Marmur, J. & Doty, P. (1959). *Nature*, **183**, 1429.
Weibull, C. (1958). *Ann. Rev. Microbiol.* **12**, 1.
Zamenhof, S., Reiner, B., DeGiovanni, R. & Rich, K. (1956). *J. Biol. Chem.* **219**, 165.

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Effects of ^{32}P Decay on Enzyme Synthesis

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Cultures of *Escherichia coli* 15T⁻ inactivated by ^{32}P decay to a survival of 0·1% are capable of synthesizing phosphatase at about 25% of the rate found in completely viable cells. These cultures also retain a similar fraction of their ability to form β-galactosidase, if no energy source is present during the induction. If glycerol is added to the medium, however, no β-galactosidase is formed. The earlier observation of an apparent concomitant decline in enzyme-forming capacity and viability following ^{32}P decay can therefore be attributed to repression on the synthesis of catabolic enzymes in cells in which the rate of catabolism exceeds the rate of growth.

1. Introduction

Thymine starvation and decay of ^{32}P atoms contained in the DNA both cause progressive declines in viability of cultures of *Escherichia coli*, probably by damaging the cells' genetic structure (Barner & Cohen, 1954; Fuerst & Stent, 1956). The immediate effects of both methods of inactivation on cellular metabolism are similar, as Brock & Brock (1960) have observed. The rates of net protein and RNA syntheses begin to decline shortly after viability is lost, although the decline in RNA synthesis is somewhat slower than that of protein synthesis, and eventually both rates approach zero. Furthermore, capacity for formation of several catabolic enzymes declines at about the same rate as viability (McFall, Pardee & Stent, 1958). These results suggested that a cell that had suffered irreparable damage to its DNA could no longer synthesize specific proteins, that is, that the structural integrity of the genetic apparatus as a whole is essential to the synthesis of functional enzymes. The observation that thymineless death does not immediately inhibit phosphatase formation, however, indicated that this might not be true (McFall & Magasanik, 1960). It suggested rather that the decline in protein synthesis in cells not capable of replication led to an accumulation within these cells of the intermediate products of catabolic reactions, which, as shown by earlier observations (Magasanik, Neidhardt & Levin, 1958), would repress the further formation of catabolic enzymes. When the energy source, glycerol, was removed from the growth medium of a culture inactivated by thymine starvation, the cells were able to synthesize β-galactosidase (McFall & Magasanik, 1960). In view of this result it seemed advisable to determine whether the loss of enzyme-forming capacity observed in cells inactivated by ^{32}P decay was not also due to such a repression. The results described below indicate that such is indeed the case.

2. Materials and Methods

The strains of *E. coli* utilized in this study were 15T⁻PA⁻, a thymine- and phenylalanine-requiring mutant; strain 3000, capable of forming β-galactosidase on induction, and 3300, a strain of K12 forming β-galactosidase constitutively.

The cells were cultivated in H medium, the glycerol-casamino acids medium described previously (McFall *et al.*, 1958). For these experiments lactate was omitted from this medium. When formation of phosphatase was desired the usual casein hydrolysate was replaced by the phosphate free preparation. For studies of enzyme formation in the absence of an energy source, glycerol was omitted.

Cultivation of cells in radioactive medium, bacterial assays (Fuerst & Stent, 1956), and biochemical assays for β-galactosidase (Pardee, Jacob & Monod, 1959), phosphatase (Torriani, 1960), protein (Lowry, Rosebrough, Farr & Randall, 1951) and RNA (Schneider, 1945), were performed as described in the literature.

3. Experimental

(a) *Effects of ^{32}P decay on the formation of phosphatase*

The following experiment was performed to determine whether cells inactivated by ^{32}P decay are capable of synthesizing phosphatase. H medium of specific activity 50 mc/mg P was inoculated to a cell density of 10^6 per ml. from a growing culture of

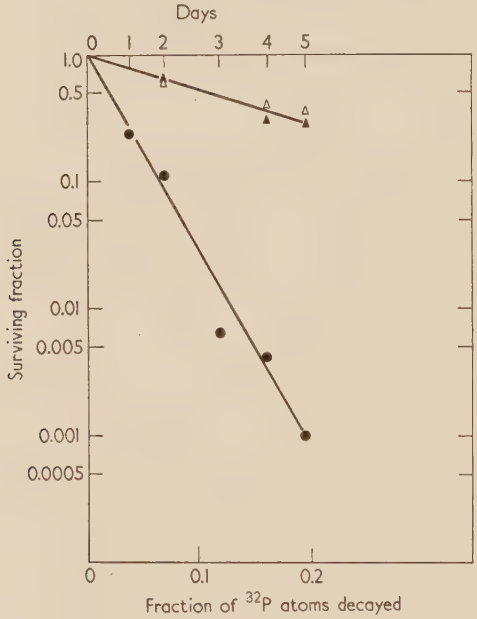

Fig. 1. Inactivation by ^{32}P decay of viability and phosphatase-forming capacity. Ordinate: fraction of the bacterial population capable of forming a colony, or ratio of the amount of enzyme formed per culture portion on *n* days to that formed on *o* days. Viability, ●–●. Enzyme formed in 45 min, ▲–▲. Enzyme formed in 60 min, △–△.

E. coli 15T⁻. This radioactive culture was permitted to grow to a cell density of 10^8 per ml. The cells were then centrifuged and washed once with phosphate-free H medium. They were then suspended at a cell density of 5×10^7 per ml. in phosphate-free H medium containing 3% glycerol as protection against the lethal effects of freezing and thawing, and 0·5 ml. portions were frozen and stored in liquid nitrogen. From day to day as ^{32}P decay progressed portions were thawed as rapidly as possible at 37 °C, assayed for residual viability, and allowed to form phosphatase for 30, 45 and 60 minutes. The results are presented in Fig. 1. It seems clear that phosphatase formation is quite resistant to ^{32}P decay, for by the time viability had fallen to 0·1%, the ability to synthesize the enzyme had fallen only to 25% of its original value.

(b) *Effects of ^{32}P decay on the formation of β-galactosidase*

To measure the actual effect of ^{32}P decay on β-galactosidase-synthesizing capacity it was necessary to perform the induction in the absence of an energy source. Glycerol-free H medium proved to be an excellent medium for this purpose. The casamino acids that it contains apparently do not serve as an energy source, but do permit β-galactosidase synthesis to proceed in viable cells at about the same rate as in the complete medium, for at least 45 minutes. For the experiments described below, therefore, the omission of glycerol during induction did not result in a depressed rate of β-galactosidase synthesis.

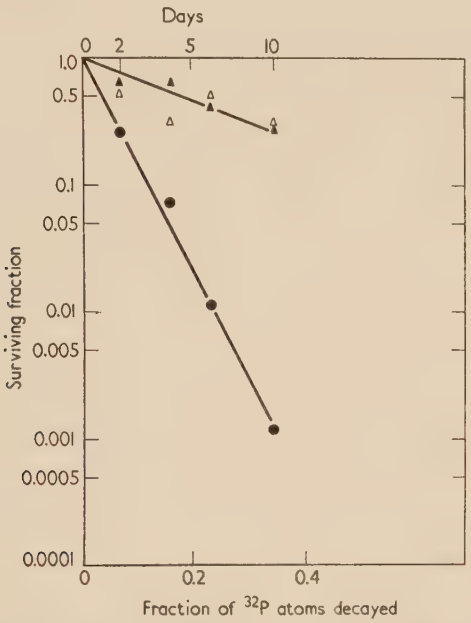

FIG. 2. Inactivation by ^{32}P decay of viability and β-galactosidase-forming capacity. Ordinate: as in Fig. 1. Viability, ●–●. Enzyme formed in 25 min, ▲–▲. Enzyme formed in 35 min, △– △.

A radioactive culture of *E. coli* 15T⁻ of specific activity 50 mc/mg P was grown as described above, centrifuged, and washed with glycerol-free H medium. It was then suspended at cell density 5×10^7 per ml. in glycerol-free H medium containing 6% ethylene glycol as protection against the lethal effects of freezing and thawing, and 0·2 ml. portions were frozen and stored in liquid nitrogen. 80 to 90% of the cells survived freezing and thawing in this medium, and ethylene glycol did not repress β-galactosidase formation in a culture inactivated by thymine starvation. (12% sucrose is also an excellent protective agent, but contains impurities that were found to repress enzyme formation in non-viable bacteria.) It was thus possible to induce the cells to form β-galactosidase in the medium in which they had been frozen, since it contained no readily utilizable energy source. From day to day, as ^{32}P decay progressed, samples were thawed, assayed for residual viability, and induced to form β-galactosidase for 15, 25 and 35 min. The results are presented in Fig. 2. These results are quite similar to those of the preceeding experiment; by the time viability had declined to 0·1%, enzyme-forming capacity had fallen only to 25%. When glycerol was added to a

final concentration of 6% to cells of residual viability 0·1%, and the cells were then induced for 25 and 35 min, however, no enzyme was formed. The striking reduction in enzyme-forming capacity observed to occur with progressive ^{32}P decay in previous work thus appears to have been due almost entirely to repression. As it happened, all of the enzymes tested in those experiments were involved in energy metabolism. The slow decline in capacity for formation of enzymes observed in the present experiments is probably a function of the decline in rate of protein synthesis which occurs with progressive ^{32}P decay.

(c) *Constitutive formation of β-galactosidase*

In previous experiments it was found that capacity for constitutive synthesis of β-galactosidase in strain W 1317, a lysogenic derivative of K12, was inactivated by ^{32}P decay (McFall *et al.*, 1958). Since this strain is normally nonrepressed, it

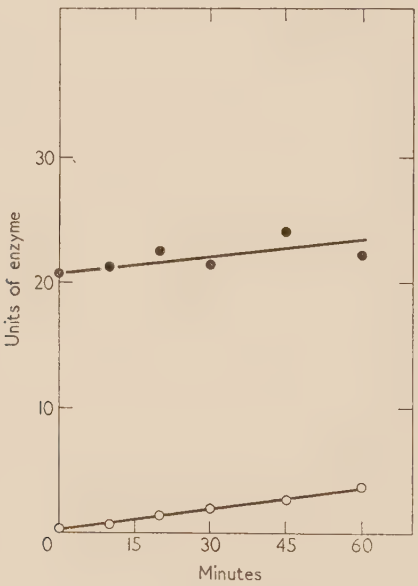

Fig. 3. β-galactosidase formation by strains 3300 (constitutive) and 3000 (inducible) in the absence of growth. Assays were performed on 0·2 ml. culture portions at cell densities of 4×10^7/ml. The final volume of the assay mixture was 1·2 ml. Enzyme content of the constitutive strain, ●–●. Enzyme content of the inducible strain, ○–○.

should not be subject to the glucose effect. There are several circumstances that may combine to explain this result, however. The exact relation between the rate of loss of enzyme-forming capacity and the rate of loss of viability is not known. These cells are lysogenic for lambda phage, and zygotic induction occurred after a small amount of ^{32}P decay, hence residual viability was not measurable; it was simply observed that enzyme-forming capacity declined by about 80% during the course of the experiment. The reduced rate of protein synthesis could have resulted in considerable reduction in the rate of enzyme synthesis, while the high background of enzyme already present in the cell made accurate measurement of increments difficult. The nature of this problem is indicated in Fig. 3. Cultures of strain 3300, which forms β-galactosidase constitutively, and 3000, the inducible parent strain, were suspended in H medium lacking both glycerol and phosphate to stop nucleic acid synthesis and

limit protein synthesis, and permitted to synthesize the enzyme for various periods. There was considerable net synthesis of β-galactosidase by the inducible strain, and probably about the same amount by the constitutive mutant. The high enzyme background in the latter case, however, tends to obscure accurate measurement of the rate of net synthesis. The results of the earlier experiments with the constitutive mutant are therefore probably not irreconcilable with the present work.

4. Discussion

The term "glucose effect" describes the repression exerted on the synthesis of catabolic enzymes in cells in which the degradation of an energy source proceeds relatively more rapidly than biosynthetic processes (Magasanik *et al.*, 1958). Growing cultures of *E. coli* and *Aerobacter aerogenes* degrade glucose about twice as fast as the breakdown products can be utilized (Neidhardt, 1960). These products accumulate within the cell and repress the formation of many catabolic enzymes, such as β-galactosidase. Glucose is exceptional in giving rise to such an accumulation under normal conditions of growth, but other substances have been found to do so when growth is limited. For example, a guanine-requiring mutant of *A. aerogenes* is unable to synthesise glycerol dehydrogenase and inositol dehydrogenase in the presence of succinate and the absence of guanine (Magasanik *et al.*, 1958). A condition analogous to the "glucose effect" should be suspected, therefore, when synthesis of catabolic enzymes does not occur in cells which are capable of energy production but not of growth.

It seems likely that a culture inactivated by ^{32}P decay might also be metabolically unbalanced. The rate of net protein synthesis declines markedly shortly after loss of viability, but there is no reason to believe that the functioning of catabolic enzymes is at all impaired. The results presented above support this idea, for a culture inactivated by ^{32}P decay can be induced to form β-galactosidase in the absence but not in the presence of an energy source. Phosphatase, an enzyme whose synthesis is not sensitive to such repression, is synthesized by inactivated cells in the presence of glycerol. The slow decline in enzyme-forming capacity which does occur with progressive ^{32}P decay probably reflects the decline in rate of protein synthesis occurring in the inactivated cells.

It is possible that the type of repression observed in these experiments may be responsible for the loss of enzyme-forming capacity observed after ^{32}P decay in initially non-radioactive recipients of radioactive chromosomal segments via $F^- \times$ Hfr mating (Riley, Pardee, Jacob & Monod, 1960) or transduction (Starlinger, 1959). If ^{32}P decay in the newly acquired fragment rendered the recipient non-viable, the effect on protein synthesis and energy metabolism should be the same as in the experiments presented here.

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REFERENCES

Barner, H. D. & Cohen, S. S. (1954). *J. Bact.* **68**, 80.
Brock, T. D. & Brock, M. L. (1960). *Bact. Proc.* **13**, 178.
Fuerst, C. F. & Stent, G. S. (1956). *J. Gen. Physiol.* **40**, 73.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265.
Magasanik, B., Neidhardt, F. C. & Levin, A. P. (1958). In *Physiological Adaptation*, p. 159. Washington, D.C.: American Physiological Society.
McFall, E. & Magasanik, B. (1960). *Biochim. biophys. Acta*, **45**, 610.
McFall, E., Pardee, A. B. & Stent, G. S. (1958). *Biochim. biophys. Acta*, **27**, 282.
Neidhardt, F. C. (1960). *J. Bact.* **80**, 536.
Pardee, A. B., Jacob, F. & Monod, J. (1959). *J. Mol. Biol.* **1**, 165.
Riley, M., Pardee, A. B., Jacob, F. & Monod, J. (1960). *J. Mol. Biol.* **4**, 216.
Schneider, W. C. (1945). *J. Biol. Chem.* **161**, 293.
Starlinger, P. (1959). *Z. Naturf.* **14b**, 523.
Torriani, A. (1960). *Biochim. biophys. Acta*, **38**, 460.

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Letter to the Editor

Some Remarks on the Morphology of Bacteriophage T4B

The use of the negative staining technique, as introduced by Hall (1955) and Huxley (1956) and made suitable for routine use by Brenner & Horne (1959), has found extensive application for the study of small particles. Especially for investigations concerned with bacteriophage morphology it has given a great deal of detailed information.

The structural components of T-even phages as revealed by the use of negative contrast technique were first described by Brenner *et al.* (1959). In a recent publication Anderson (1960) confirmed their findings and additionally described in the case of T4 phage the occurrence of a disc or collar between the base of the head and the beginning of the tail sheath.

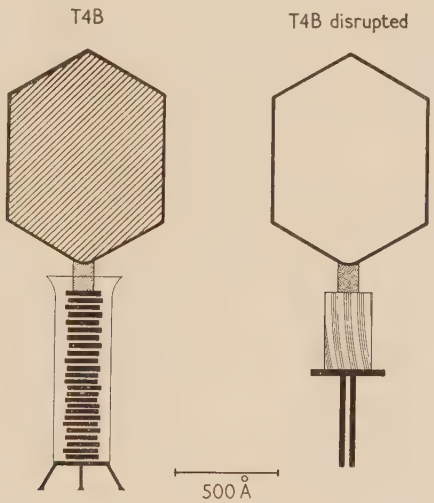

Fig. 1. Diagram of T4B phages, normal (left) and disrupted (right).

It is the purpose of this paper to add some supplementary details to the picture already known of T4 bacteriophages. In our electron micrographs made with a Siemens Elmiskop I of samples of T4 (strain T4B) embedded in phosphotungstic acid (PTA) (Brenner & Horne 1959) all previous findings have been confirmed. In our material the head of the phage particles, which commonly shows the characteristic appearance of a bipyramidal hexagonal prism, is fixed to the tail sheath by a short neck about 125 Å long and about 90 Å wide. The tail sheath reveals the typical pattern of 25 cross striations. Tail fibres could seldom be observed. In some instances, superimposed on the cross-striations of the tail sheath, a helical structure is observed which shows a varying pitch (Plate I(a)). Moreover around the tail sheath an outer jacket can be seen (Plate I(a)). This jacket, which is observed in nearly all of our samples, is schematized in Fig. 1. It extends upward from the base plate alongside the sheath. At the

point where the tail sheath ends, the jacket widens and becomes linked with the collar described by Anderson. Together with collar and base plate it appears to form one well-defined structure.†

In contrast to the T4B bacteriophage the T2 phage particles we have examined show neither collar nor jacket (Plate I(b)). They correspond with the T-even phage described by Brenner *et al.* (1959), Plate VII of their publication.

It is generally accepted that in order to inject the nucleic acid into the bacterium the pins of the base plate of the phage particle become attached to the host cell wall. Then the tail sheath contracts and finally the hollow core penetrates into the host. Such a mechanism implies that the base plate remains attached to the contracted tail sheath. Tail sheaths contract also *in vitro* after treatment with H_2O_2, alcohol (Kellenberger & Arber, 1955; Brenner *et al.*, 1959) or $Cd(CN)_3$ (Kozloff, Lute & Henderson, 1957). As could be observed in our material, mechanical damage during spraying can also induce contraction of the sheath (Plate II(c)). It appears that under these conditions the base plate is generally separated from the contracted sheath. The contraction mechanism in this case is thus quite different from that postulated for infection *in vivo*.

In 1957 Fraser, Mahler, Shug & Thomas described a method for disruption of bacteriophage T2 by means of urea. Our urea-disrupted preparations of T4B display large numbers of ghosts, and also some particles with filled heads (Plate II(a)). The tail sheaths of all the particles are contracted, thereby pulling the base plate up and leaving the greater part of the core free. The distance from the end of the core to the base of the head appears to be as long as the tail of the untreated phage. The contracted sheath has longitudinal striations, in contrast to the cross-striations in the extended sheath (Plate II(b)). These longitudinal striations may be related to the cog-wheel appearance of the cross-section of purified contracted sheaths seen by Brenner *et al.* (1959). The striations of the contracted sheaths in our T4B sample possess a helical pattern similar to that seen by Brenner *et al.* for T2 and by Anderson for P1 bacteriophages.

We conclude that the contraction caused by the disruption of the bacteriophages with urea, in contrast to other chemical or mechanical treatments, agrees well with the mode of contraction which is generally assumed to occur during the actual process of infection.

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† In this respect the opinion of Horne (in discussion at the Conference on electron microscopy, Delft, 1960), who expressed the possibility that the tail fibres might turn upwards, is of interest. It is possible that the jacket we observe consists of turned-up tail fibres. However, the form and dimensions of our jacket are constant, and appear to be independent of the position of the phage particle with respect to the optical axis of the microscope. Occasionally tail fibres are observed together with the jacket. Moreover, it seems improbable that 1300 Å tail fibres can form an 800 Å jacket with disappearance of the remainder of the fibres. The characteristic kink of the tail fibres is also absent. Finally the tail fibres appear to be attached to the pins of the base plate and not, as is the case with the jacket, to the base plate itself.

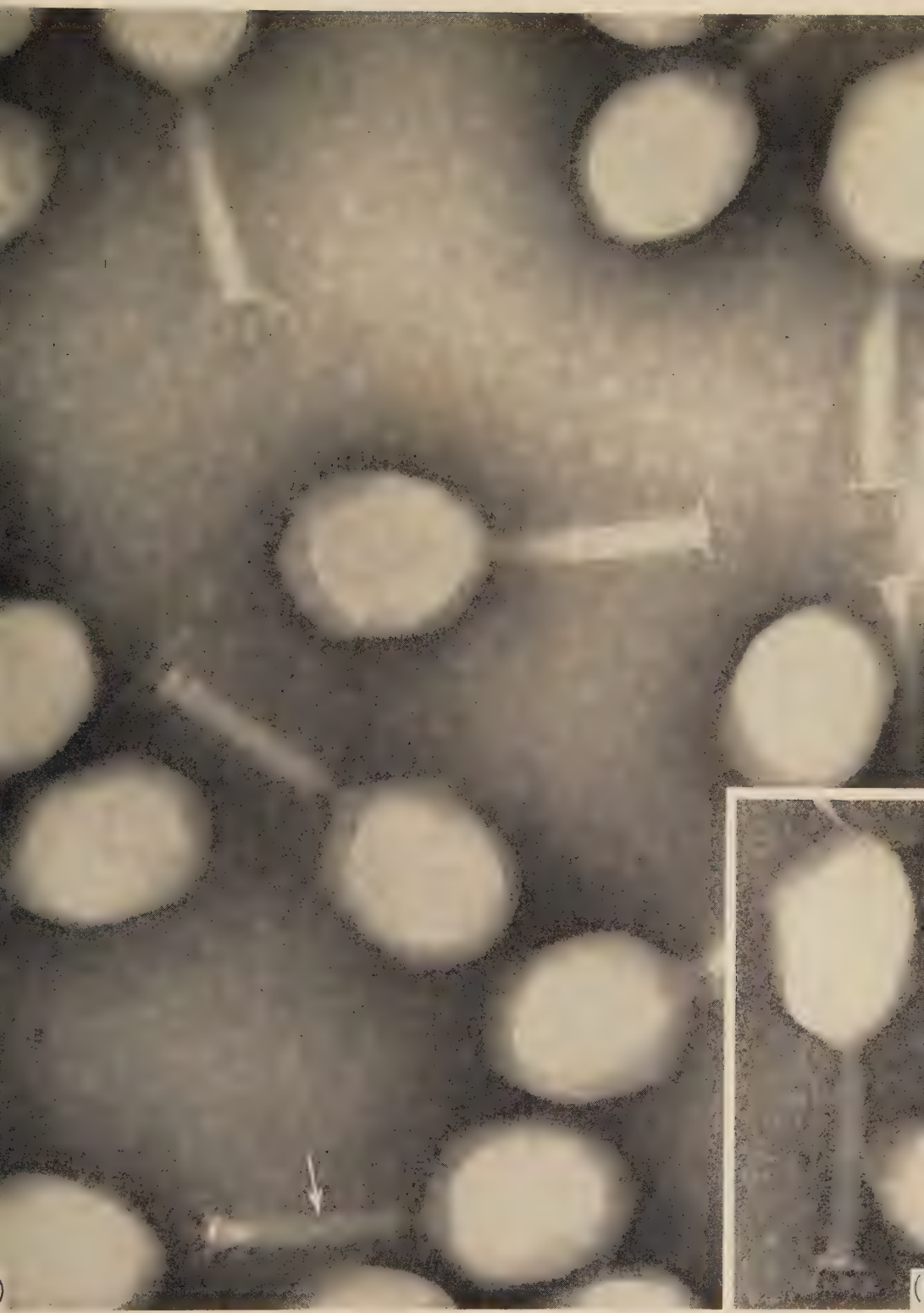

PLATE I(a) T4B phage particles showing, besides the general characteristic appearance, a jacket along the tail sheath, extending from base plate to collar. The arrow indicates helical structure superimposed on the cross-striations of the tail sheath. Often the cross-striations can be observed to extend over the whole width of the jacket. (PTA-embedded, about $\times 260{,}000$.)

(b) T2 phage particle. Neither collar nor jacket is visible. (PTA-embedded, about $\times 260{,}000$.)

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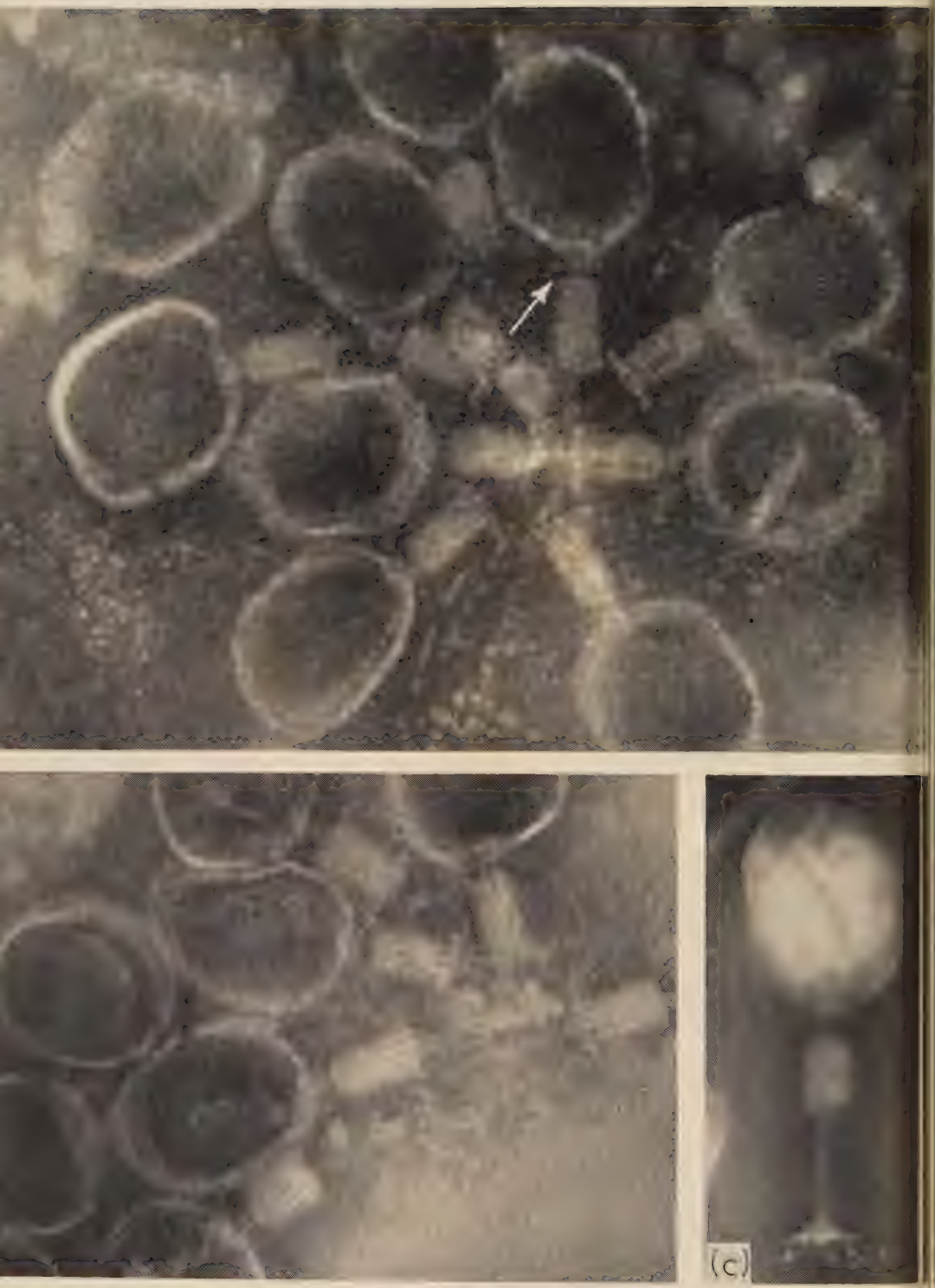

PLATE II (a) Rosette of urea-disrupted T4B phage particles. The empty heads show a preserved hexagonal shape. The base plate is still attached to the contracted sheaths. The arrow indicates possible remnants of collar. (PTA-embedded, about $\times 260{,}000$.)

(b) Urea-disrupted T4B phage particles which clearly show the longitudinal striations along the contracted sheath. (PTA-embedded, about $\times 260{,}000$.)

(c) Mechanically disrupted T2 phage particle. The base plate is separated from the contracted sheath and is attached to the end of the core. The head shows an unaltered appearance. (PTA-embedded, about $\times 260{,}000$.)

REFERENCES

Anderson, T. F. (1960). *Proc. Regional Conf. on Electron Microscopy*, Delft. In the press.
Brenner, S. & Horne, R. W. (1959). *Biochim. biophys. Acta*, **34**, 103.
Brenner, S., Streisinger, G., Horne, R. W., Champe, S. P., Barnett, L., Benzer, S. & Rees, M. W. (1959). *J. Mol. Biol.* **1**, 281.
Fraser, D., Mahler, M. R., Shug, A. L. & Thomas, C.A., Jr. (1957). *Proc. Nat. Acad. Sci., Wash.* **43**, 939.
Hall, D. (1955). *J. Biophys. Biochem. Cytol.* **1**, 1.
Huxley, H. E. (1956). *Proc. Regional Conf. on Electron Microscopy, Stockholm*, p. 260. Almqvist & Wiksell.
Kellenberger, E. & Arber, E. (1955). *Z. Naturf.* **106**, 698.
Kozloff, L. M., Lute, M. & Henderson, K. (1957). *J. Biol. Chem.* **228**, 511.

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